Analysis and biochemical characterization of degradation products of anti-Alzheimer's drugs

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2011

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Publisher's PDF, also known as Version of record

Chapter 3

Production and on-line acetylcholinesterase bioactivity profiling of chemical and biological degradation products of tacrine

Adapted from:
Lygia Azevedo Marques, Jeroen Kool, Frans de Kanter, Henk Lingeman, Wilfried Niessen and Hubertus Irth. *Production and on-line acetylcholinesterase bioactivity profiling of chemical and biological degradation products of tacrine.* Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 609-616.

Abstract

The work describes a methodology for rapid assessment of chemical and biological degradation products of tacrine and their bioactivity for acetylcholinesterase (AChE). Analysis was achieved by utilizing liquid chromatography coupled to parallel high resolution mass spectrometry and an on-line continuous-flow AChE bioassay for biochemical detection. Key advantage of the strategy described involves the straightforward chemical production of large quantities of products of which many were the same as formed during the biological degradation by cytochromes P450 (CYPs). For this, chemical degradation of tacrine was evaluated under acidic, basic and oxidative conditions as well as elevated temperatures and light exposure. Chemical degradation products were only formed after 2 h under reflux with 3% hydrogen peroxide, where more than 50% of tacrine was converted to degradation products. Many of these products showed bioactivity. Mostly, mono-, di- or tri-oxygenated compounds were observed. This study demonstrated that the combination of chemical and biological degradation provides valuable information indicating that assessment of biological activity is important not only for biological degradation products, but also for chemical degradation products when formed. Furthermore, chemical degradation can be used to produce conveniently and in relatively large quantities clean mixtures of compounds that are also produced during metabolic incubations.

3.1 Introduction

The enzyme acetylcholinesterase (AChE) is present in the synapse between nerve and muscle cells and is responsible for breaking down acetylcholine (Talesa, 2001). Studying AChE is important due to its relation with Alzheimer's disease (AD), which is reflected by the fact that most AD drugs inhibit AChE (Tabet, 2006, Yu *et al.*, 2008). The use of AChE inhibitors for AD treatment started in the mid 1980s with tetrahydroaminoacridine (tacrine) being the first drug approved by the FDA for the treatment of AD (Crismon, 1994, Davis and Powchik, 1995, Knapp *et al.*, 1994). Chemical degradation studies have been performed to evaluate the safety, efficacy and quality of tacrine (ICH, 2003). Depending on ICH guidelines and the final goal for degradation studies, most of the tests performed include exposure to extreme acidic, basic, oxidative conditions, as well as elevated temperatures, humidity and light (Bakshi and Singh, 2002).When chemical degradation studies are performed, usually the presence of degradation products and their chemical structure are looked at, but their biological activity is not investigated. However, biological degradation in the body after metabolism by, e.g., cytochromes P450 (CYPs) has been investigated by many studies for tacrine (Bao *et al.*, 2002, Hansen *et al.*, 1998, Hsu *et al.*, 1990, Madden *et al.*, 1993, Pool *et al.*, 1996). In some of these studies, the biological activities of metabolites were the focus as the metabolites might contribute to the overall biological effect. For these studies, however, cumbersome purification steps are necessary to investigate the biological effects of individual metabolites formed. Direct, rapid and sensitive bioactivity assessment of metabolites in combination with their identification would be preferable. These characteristics can be achieved using mass spectrometry in parallel with on-line continuous-flow biochemical detection after separation (Elswijk *et al.*, 2003). Furthermore, straightforward (chemical) production of large quantities of compounds that are also formed during biological degradation by CYPs (metabolites) would be advantageous as it prevents bottlenecks for further studies towards these metabolites.

The present study describes the efficient and comprehensive analysis of chemical and biological degradation products of tacrine involving their simultaneous identification and bioactivity assessment. One of the key features of the study involves the chemical (and UV) production of oxidation products, which can be used as substitutes for metabolites formed during biological degradation by CYPs. For the study, the separation power of HPLC was coupled on-line in parallel to high resolution mass spectrometry (HRMS) and an on-line fluorescence-based AChE bioassay (Rhee *et al.*, 2003). The system allows direct bioactivity assessment towards AChE and identification of all eluting compounds in a single chromatographic run. It is important to emphasize that at this stage the methodology is used for (initial) in vitro screening purposes only (with the target enzyme AChE). A qualitative comparison between chemical and biological degradation products was made. Furthermore, for illustration purposes, the chemical degradation production was scaled up to allow identification with NMR of compounds formed.

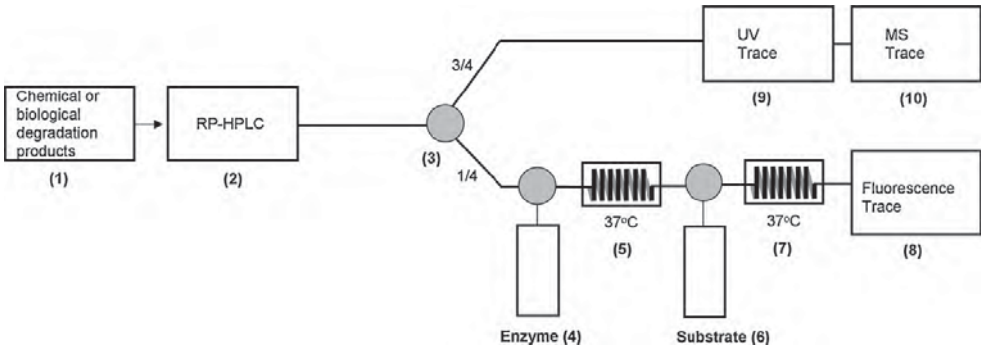


Figure 3.1 Scheme of the on-line AChE bioactivity profiling system. The system was utilized for the analysis of chemical and biological degradation products of tacrine with parallel high resolution mass spectrometry (HRMS): (1) injection of mixtures with chemical or biochemical degradation products, (2) analytical separation of chemical and biological degradation products by RP-HPLC followed by a split (3) to chemical identification by UV (9) and HRMS (10) and split to the on-line AChE bioassay, where enzyme (4) and substrate (6) are continuously pumped into a reaction coil of 100 μL at 37 °C (5 and 7) where the reaction enzyme/substrate takes place. The signal is monitored by a fluorescence detector (8).

3.2 Experimental

3.2.1 Chemicals

All solvents used were of HPLC grade. Methanol (MeOH) was purchased from Biosolve (Valkenswaard, the Netherlands), formic acid and hydrogen peroxide were obtained from Merck (Zwijndrecht, the Netherlands and Darmstadt, Germany, respectively). Milli-Q water was obtained from a Milli-Q purification system (Millipore, Bedford, MA, USA). Tacrine hydrochloride, sodium hydroxide, hydrochloric acid, bovine serum albumin (BSA), citric acid, magnesium chloride, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and acetylcholinesterase type VI-S from *Electrophorus electricus* (electric eel) were purchased from Sigma (Zwijndrecht, the Netherlands). 7-Acetoxy-1-methyl-quinolinium iodide (AMQ) was supplied by Fluka Chemie GmbH (Buchs, Switzerland). The enzyme and the substrate stock solutions were kept in −80 °C until use.

3.2.2 System overview

In Figure 3.1, an outline of the setup of the system is shown. The instrumentation consists of two isocratic Agilent 1100 series HPLC pumps (Agilent Technologies, Waldbronn, Germany) controlled by one gradient LC setup and a Gilson Model 364 auto-sampler (Gilson, Villiers Le Bel, France). For chemical detection, an Agilent 1100 series UV diode array detector (200–400 nm) and an Agilent Q-TOF HRMS were used in series. Two LC-pumps from LabAlliance (Nieuwegein, the Netherlands) controlled the two house-made superloops filled with either

enzyme or substrate solution, resulting in the continuous delivery of enzyme and substrate to the on-line AChE bioassay. The effluent from the analytical column was split to 187.5 µL to the mass spectrometer for chemical detection and 62.5 µL to the on-line AChE bioassay. In the present study, a fluorescence-based AChE bioassay based on the work of Rhee *et al.* (Rhee *et al.*, 2003) was used because it is straightforward and robust. The on-line AChE bioassay was monitored by an Agilent 1100 series fluorescence detector with the excitation wavelength set at 406 nm and the emission wavelength set at 505 nm.

3.2.3 HPLC conditions

For the analytical separation, a Waters Symmetry shield 2.1 mm × 100.0 mm C_{18} column with 3.5 µm particles and stainless steel filters (Waters, Milford, MA, USA) was used. The analytes were eluted at a flow-rate of 250 µL/min. The column oven was set at 30 °C. Solvent A consisted of H_2O–MeOH–formic acid (97:9:2:0.1, v/v/v) and solvent B consisted of H_2O–MeOH–formic acid (10:89.9:0.1, v/v/v). Gradient analysis runs started with 0% solvent B for 7 min, followed by a linear increase to 35% solvent B in 30 min, followed by an increase to 100% solvent B in 1 min, where it is kept for 3 min, before returning to initial conditions in 1 min. The total run time is 43 min. The sample volume injected was 50 µL.

3.2.4 Mass spectrometry conditions

Chemical and biological degradation products were identified using an Agilent Q-TOF mass spectrometer model number 6520. Analyses were performed with electrospray ionization (ESI) in the positive ion mode. The following conditions were used — interface voltage: 4.0 kV, nebulizer gas (N_2): 40 psi, desolvation gas (N_2): 8 l/min and source temperature: 350 °C. Spectra were acquired in the range of *m/z* 50–500. MS/MS experiments were performed with an isolation width set to medium (~ 4 *m/z*-units wide) and collision energy of 35 eV. High resolution measurements were acquired after tuning and instrument calibration according to specifications.

3.2.5 On-line acetylcholinesterase conditions

The on-line bioassay used was a slightly modified version of the one developed by Rhee *et al.* (Rhee *et al.*, 2003). The binding between AChE and eluting compounds in the AChE bioassay took place in a reaction coil of knitted 0.25 mm i.d. peek tubing with a volume of 100 µL at 37 °C. AChE and substrate 7-acetoxy-1-methyl-quinolinium iodide are continuously pumped into the bioassay, resulting in the continuous production of the highly fluorescent 7-hydroxy-1-methyl-quinolinium product (HMQ) (Rhee *et al.*, 2003), when eluting active compounds bind to the enzyme, the formation of the highly fluorescent HMQ does not take place resulting in a negative peak in the bioassay chromatogram. The AChE (0.1 U/L) and substrate (1 µM) solutions were delivered at a flow-rate of 70 µL/min to the on-line bioassay via 100 mL superloops made in-house. The buffer used for the AChE solution was a sodium phosphate buffer (pH 7.0; 0.1 M) with 0.1 mg/mL of BSA and the buffer used for the substrate solution was a sodium phosphate/citrate buffer (pH 5.0; 0.1 M).

3.2.6 Chemical degradation

Chemical degradation studies were performed according to ICH guidelines (ICH, 2003). The following conditions were evaluated with a stock solution of 6.3 mM of tacrine: acidic, alkaline and oxidative conditions as well as elevated temperatures and light exposure. Initially, only LC-UV (wavelengths of 210, 220, 240, 254 and 280 nm) was used to evaluate possible degradation products. When degradation products were found, the on-line AChE bioassay system was subsequently applied in order to study the bioactivity of the chemical degradation products. The chromatograms (UV, MS and AChE bioassay) were time-aligned by the Agilent ChemStation software, taking the retention time of tacrine as a reference. The experiment was performed in duplicate with three injections of each sample.

3.2.6.1 Acid and alkaline hydrolysis

A solution of 0.3 mM of tacrine was refluxed in either 2 M HCl or 2 M NaOH for acid and alkaline hydrolysis, respectively, for 5 and 10 h. Aliquots of 250 µL were taken, neutralized and diluted to 500 µL. A control sample in water was refluxed under the same conditions. Aliquots of 250 µL were taken and diluted to 500 µL. Aliquots of 50 µL were injected into the HPLC-UV.

3.2.6.2 Oxidation

A solution of 1.2 mM of tacrine was refluxed with 3% of hydrogen peroxide for 2 h. Also, a control sample in water was refluxed at the same conditions. Aliquots of 100 µL were taken and diluted to 400 µL. Aliquots of 50 µL were injected into the HPLC-UV. The up scaling of the chemical degradation was slightly modified compared to the analytical scale. Here, 2 mM of tacrine was used in a total volume of 250 mL. From this, 100 mL was injected over 20 injections of 5 mL onto a self-packed column (2 cm × 30 cm) with Phenomenex Luna C_{18}(**2**), 10 µm material. Then, a gradient from 100% solvent A to 100% solvent B in 90 min (3 mL/min) was applied and fractions of 2 min were collected. From there, some fractions were applied to NMR for demonstration of the methodology to obtain sufficient compound for NMR based analysis.

3.2.6.3 Light exposure

Sample and control solutions of a concentration of 0.3 mM of tacrine in water in quartz cuvettes were exposed to a xenon lamp (150 W) with an appropriate filter fitted to eliminate radiation below 320 nm. The control sample was protected from light with aluminium foil. Aliquots of 250 µL were taken and diluted to 500 µL. Aliquots of 50 µL were injected into the HPLC-UV.

3.2.6.4 Temperature exposure

A solution of 0.3 mM of tacrine in water was placed for 2, 5 and 7 days at 60 °C. A control sample in water was maintained at room temperature. Aliquots of 250 µL were taken and diluted to 500 µL. Aliquots of 50 µL were injected into the HPLC-UV.

3.2.7 NMR data

NMR spectra from a typical fraction containing a ketone according to MS analysis were recorded on a Bruker Avance 500, equipped with a cryoprobe. Chemical shifts are reported in parts per million (ppm) and coupling constants J are given in Hertz (Hz).

3.2.8 Biological degradation

Rat liver microsomal and pig liver microsomal incubations were carried out in total volumes of 500 µL. The reaction mixtures consisted of 2.5 mM $MgCl_2$ in potassium phosphate buffer (pH 7.4; 0.1 M) and 10% of rat or pig liver microsomes (Kool *et al.*, 2006). A final concentration of 100 µM of tacrine was used for all incubations. The incubations were initiated by addition of a NADPH regenerating system, resulting in a final concentration of 0.1 mM NADPH, 0.3 mM glucose-6-phosphate and 0.4 U/mL glucose-6-phosphate dehydrogenase at 37 °C for 60 min. The incubations were terminated by addition of 1 mL of cold acetonitrile. Following centrifugation at 14,000 rpm for 5 min, the supernatants were taken, evaporated with a vacuum-centrifuge until dryness and dissolved in 200 µL of Milli-Q water for analysis. The chromatograms (UV, MS and AChE bioassay) were time-aligned by the Agilent ChemStation software, taking the retention time of tacrine as a reference. The experiment was performed in duplicate with two injections of each sample.

3.3 Results and discussion

This study deals with the on-line analysis of chemical and biological degradation products of tacrine. Furthermore, the use of chemically produced degradation products was evaluated for its value to conveniently produce larger quantities of compounds also formed during biological degradation (CYP based metabolism). The system used to study the degradation products of tacrine included HRMS for identification of degradation products while the associated AChE bioactivity was simultaneously assessed in a parallel on-line bioassay. The development of the current approach consisted of a preliminary investigation of chemical degradation of tacrine with different acidic, basic and oxidative conditions, elevated temperatures and light exposure. The tacrine degradation products were analyzed by HPLC-UV. When considerable amounts of degradation products were observed, the validated on-line AChE bioassay in parallel with HRMS (and UV) was applied. In case of biological degradation by CYPs, it was decided to analyze the resulting metabolic incubations immediately with the complete system since the formation of biologically active metabolites has already been reported in literature (Bao *et al.*, 2002, Hansen *et al.*, 1998, Hsu *et al.*, 1990, Madden *et al.*, 1993, Pool *et al.*, 1996).

The feasibility to use chemical degradation for the generation of metabolites or other (bioactive) degradation products was investigated by comparing the profiles of products formed. Finally, the methodology was evaluated for use as an alternative to generate enough material for further (structural elucidation) studies, e.g., using NMR spectroscopy.

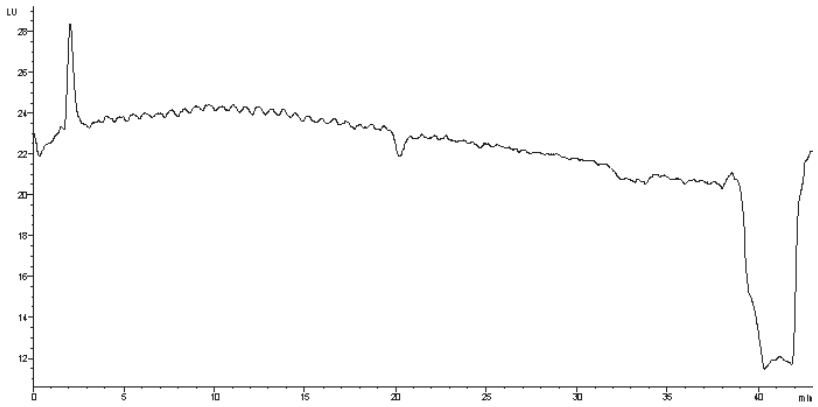


Figure 3.2 AChE bioassay chromatogram of injection of 50µL of 0.1µM of tacrine.

3.3.1 Bioassay performance

The performance of the bioassay was tested for three consecutive days with a calibration curve (in quadruplicate) of six different concentrations of tacrine (0.1, 0.5, 1.5, 2.5, 10, 25 µM). The total time for a 1-day experiment was 21 h. The %RSD value was calculated for each concentration for the 3 days. The values vary from 5.8% for the highest concentration to 35% for the lowest concentration, which actually is the limit of detection (see Table 3.1). The %RSD was calculated based on the peak height of each compound concentration (Table 3.1). The Relative Fluorescence (RF) was calculated by the peak height applying the integration for negative peaks accessible in the software of Agilent ChemStation. As the bioactivity signal is a sigmoidal-dose response one, saturation of the AChE enzyme will result in a maximal inhibitory signal (around 12 in this case) at high concentrations of tacrine or other inhibitors. By serial dilution of the samples followed by re-injections, for all bioactive compounds and their metabolites, on-line sigmoidal-dose response signals could be measured and plotted.

The limit of detection (LOD) for the bioassay was calculated based on three times the average of noise observed for four injections of blank. The value obtained corresponds to the minimal amount of tacrine that can be detected, and was 0.06 µM (see Figure 3.2 for the bioactivity chromatogram).

The baseline remained constant for at least 21 h (that is as long as substrate and enzyme solution are available in the superloops), thus proving that enzyme and substrate still functioned properly and showed no degradation during 21 h of storage in the superloops.

During separations, the maximum amount of methanol in the reaction coil of the on-line AChE bioassay was 10%. The HPLC gradient applied did not interfere with the performance of the enzymatic activity. Rhee *et al.* (Rhee *et al.*, 2003) observed that the enzyme AChE can even handle up to 30% of MeOH with moderate losses of activity (~30%), which corresponds well with our findings.

Table 3.1 Bioassay performance measured for three consecutive days with six different concentrations of tacrine (0.1-25 µM). The peak activities were measured by the height of the negative peaks using Agilent Chemstation software.

Day	Repetition	Concentration (µM)					
		0.1	0.5	1.5	2.5	10	25
1	1	2.0	2.8	4.5	5.8	9.3	11.1
	2	4.3	3.6	4.3	5.4	9.2	10.7
	3	3.7	3.6	4.3	5.5	8.8	10.6
	4	4.0	3.1	4.1	5.1	8.6	10.1
%RSD		29.1	12.1	2.9	5.3	3.7	3.9
2	1	1.1	2.1	4.1	5.6	9.8	11.9
	2	2.4	2.6	4.2	5.6	9.6	11.2
	3	2.3	2.5	4.3	5.7	9.3	11.2
	4	1.7	2.6	4.1	5.2	9.0	10.8
%RSD		32.1	9.7	2.3	4.0	3.7	4.1
3	1	1.4	2.4	4.7	6.2	10.6	12.5
	2	3.2	3.1	4.7	6.7	9.9	11.8
	3	2.4	2.6	4.7	5.8	10.1	11.5
	4	2.3	3.5	4.3	5.4	10.0	11.3
%RSD		31.7	17.2	4.4	9.2	3.1	4.4

3.3.2 Chemical degradation studies

Chemical degradation of tacrine was evaluated under the following conditions: 2 M of hydrochloric acid or 2 M of sodium hydroxide both for 10 h under reflux, 3% of hydrogen peroxide (H_2O_2) for 2 h under reflux, 60 °C over a period of 7 or 2 days under UV-light exposition. Chemical degradation products were not observed under acid and alkaline conditions or at elevated temperatures. Similar results were observed by Sathyan *et al.* who investigated the stability of tacrine at room temperature, 37 °C in water and in phosphate buffers at pH 1.3; 3.2; 5.5; 6.7; 7.8 and 8 (Sathyan *et al.*, 1995). These solutions were analyzed on different days for a period of 30 days and tacrine was found to be stable at all conditions. Wilson *et al.* obtained comparable results when investigating the chemical degradation of tacrine with poly(*n*-butylcyanoacrylate) stored at room temperature (15–20 °C), refrigerated (3–5 °C) or at 37 °C (RH: 75%) over a period of 3 months (Wilson *et al.*, 2008). Tacrine and the polymer were found to be physically and chemically stable and retained their pharmaceutical properties under these conditions over a period of 3 months.

On the other hand, after light exposure or under 3% of H_2O_2, our study showed that tacrine was not stable. After 2 days under UV-light exposition, formation of two degradation products

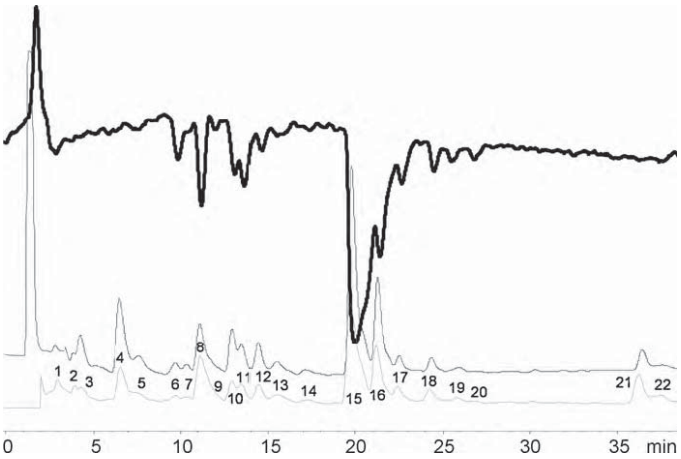
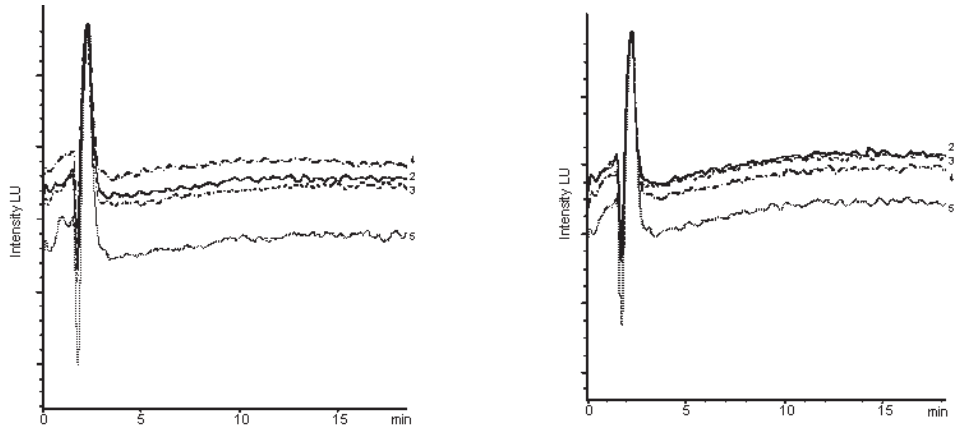


Figure 3.3 On-line AChE profiling of hydrogen peroxide chemical degradation with parallel HRMS and UV. The AChE bioassay for a sample after 2 h under reflux is presented by the upper full line chromatogram. The UV chromatogram for a sample after 2 h under reflux is presented by the lower black full line chromatogram. The MS total ion current for a sample after 2 h under reflux is presented by the lower grey full line chromatogram. Compounds numbered by **6–12** and **16–20** are bioactive degradation products of tacrine (**15**). Blank showed only one active peak corresponding to tacrine.

was observed corresponding to less than 10% tacrine degraded. Two hours under reflux with 3% H_2O_2 yielded more than 50% degradation of tacrine into several degradation products. For testing the bioactivity of the chemical degradation products, these samples were analyzed with the on-line AChE bioactivity profiling with parallel HRMS. For chemical degradation studies with UV-light, two mono-oxygenated degradation products (*m/z* 215.1170 and 213.1020) were found, which were bioactive and were also observed in the H_2O_2 degradation studies. Therefore, we focused our further investigation on the degradation products formed by H_2O_2. The bioactivity profile of the H_2O_2 chemical degradation products is depicted in Figure 3.3. In this figure, the on-line AChE bioassay trace after 2 h under reflux (upper full line), the UV trace at 240 nm (lower black line) and the MS total ion current (TIC) chromatogram after 2 h under reflux (lower grey line) are shown. Twelve bioactive degradation products were observed. The positive peak in the bioassay trace is the injection peak in the bioassay chromatogram. This phenomenon is caused mainly by the samples which were diluted in water. This temporarily increases the activity of the AChE enzyme and thus the fluorescent product formation. Furthermore, non-retained compounds showing autofluorescence and/or traditional injection peaks can be additional causes of the positive peak. In Table 3.2, a summary of the averaged retention times, their %RSD, the averaged corresponding bioactivities when applicable, their %RSD and corresponding *m/z* values of the protonated molecules and their main fragments in MS/MS are presented. Furthermore, the most likely molecular formulas of the protonated



(I) (II)

Figure 3.4 AChE bioassay chromatograms of different blank incubations of rat (I) and pig (II) CYPs. Chromatograms are shown from 0-18 minutes. Tacrine elutes at 19.5 minutes and is not shown in the Figures. The different blanks are measured in order to investigate whether (bioactive) metabolites could be formed chemically during incubation. For this, different experiments were conducted in which one or two components (NADPH, tacrine, CYPs) were omitted in every experiment. In Figures (I) and (II) the chromatograms numbered 2 represent the incubations without CYPs, chromatograms numbered 3 are incubations without NADPH and CYPs, chromatograms numbered 4 are incubations without NADPH and finally chromatograms numbered 5 are the blank incubations without tacrine. In all cases no bioactivity was observed for rat or pig liver microsomal incubations.

molecules with error margin in ppm as predicted by Agilent MassHunter Qualitative Analysis software are shown. These predicted molecular formulas indicate degradation products with one up to three incorporated oxygen atoms. Detailed structural elucidation of (some of) the chemical degradation products is described in Section 3.3.4.

3.3.3 Biological degradation products

Blanks of CYP incubations were performed in order to investigate whether metabolites could be formed chemically during incubation. For this, different experiments were conducted in which one or two components were omitted in every experiment. Details of these experiments can be found in the Figure 3.4. In none of these control experiments, any metabolites were formed.

In Figure 3.5 the AChE bioactivity chromatograms are shown for tacrine incubations with rat (upper figure) and pig (lower figure) CYPs after 1 h of incubation. The upper chromatogram (full line) is the bioactivity trace, whereas the lower chromatograms in both figures are the UV traces at 240 nm (black lines), the MS TIC chromatograms for 1 h of incubation (grey lines). The AChE bioactivity chromatograms showed active metabolites. Corresponding MS and UV data were used for the identification of the bioactive metabolites.

Table 3.2 Summary of chemical degradation compounds from tacrine formed after 2 h under reflux with 3% hydrogen peroxide. The averaged retention time, %RSD, the average corresponding bioactivities (when found) with %RSD and corresponding *m/z* values of molecular ions and their fragments of chemical degradation compounds from tacrine are shown. Moreover the probable molecular formulas with error margin in ppm predicted by Agilent MassHunter Qualitative Analysis software are also shown.

Peaks	RT average n=6	%RSD n=6	Activity average n=6	%RSD n=6	MS *m/z*	MS/MS *m/z*	Most likely molecular formula	Error (ppm)
1	2.85	0.35	-	-	-	-	-	-
2	4.24	0.37	-	-	-	-	-	-
3	5.08	0.50	-	-	-	-	-	-
4	6.50	0.23	-	-	237.0857	-	$C_{11}H_{13}N_2O_4^+$	5.5
5	7.60	0.35	-	-	237.0855	-	$C_{11}H_{13}N_2O_4^+$	6.5
6	10.10	1.09	2.1	12.5	215.1167	197.11;182.08	$C_{13}H_{15}N_2O^+$	5.5
7	10.58	0.94	0.9	20.8	215.1163	-	$C_{13}H_{15}N_2O^+$	7.3
8	11.40	1.15	4.9	3.9	213.1013	195.09;183.09; 158.08	$C_{13}H_{13}N_2O^+$	4.7
9	12.07	2.5	0.6	18.5	231.1111	-	$C_{13}H_{15}N_2O_2^+$	7.4
10	13.30	1.02	3.6	31.7	231.1119	213.10; 185.11; 158.08	$C_{13}H_{15}N_2O_2^+$	4.3
11	13.85	0.88	4.2	27.9	231.1117	213.10;185.11; 158.08	$C_{13}H_{15}N_2O_2^+$	4.7
12	14.79	1.03	1.1	23.3	247.1067	229.09;213.10; 201.10	$C_{13}H_{15}N_2O_3^+$	4.6
13	16.05	0.66	0.5	9.9	247.1066	229.09; 213.10; 201.10	$C_{13}H_{15}N_2O_3^+$	4.7
14	17.01	0.25	-	-	247.1061	-	$C_{13}H_{15}N_2O_3^+$	6.8
15	20.17	0.96	12.1	2.3	199.1216	182.08;171.09; 144.08	$C_{13}H_{15}N_2^+$	6.0
16	21.51	1.12	7.6	12.3	215.1169	199.09;187.09; 174.08;160.07	$C_{13}H_{15}N_2O^+$	4.3
17	22.90	0.60	3.0	17.0	215.1166	199.08;187.09; 174.08;160.07	$C_{13}H_{15}N_2O^+$	6.0
18	24.73	0.47	1.4	11.0	215.1164	199.08;187.08; 174.07;160.07	$C_{13}H_{15}N_2O^+$	7.0
19	25.77	0.06	0.6	30.6	-	-	$C_{13}H_{15}N_2O^+$	-
20	26.98	0.49	0.9	19.5	-	-	$C_{13}H_{15}N_2O^+$	-
21	36.40	0.04	-	-	271.1036	-	-	-
22	37.72	0.15	-	-	271.1035	-	-	-

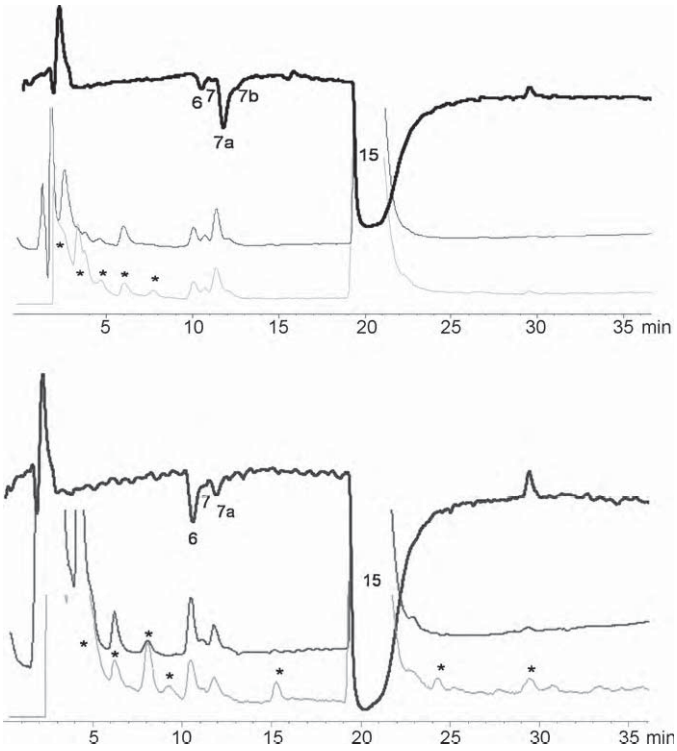


Figure 3.5 On-line AChE profiling of biological degradation. The upper and lower figures show bioactive metabolites formed after 1 h incubation with rat and with pig liver microsomes, respectively. Parallel MS total ion currents (grey lines) and UV (black lines) traces are shown as the lower two traces in both figures. The on-line AChE bioassays for samples are presented by the upper black lines in both figures. Compounds numbered by **6** and **7**a are bioactive metabolites that were also found for the chemical degradation of tacrine (**15**) and metabolites with an asterisk did not show bioactivity. Compounds **7** and **7**b show signals around the detection limit for the bioassay.

In Table 3.3, a summary of the average retention times, their %RSD, the average bioactivities when applicable, their %RSD, and the *m/z* values of the MS and MS/MS experiments are shown. The most likely molecular formulas with error margin in ppm are also shown. The table shows that the same active metabolites were formed in both rat and pig liver microsomes, except compound **7**b, which was observed in rat, but not in pig liver microsomes. Furthermore, other differences in rates of metabolite formation are reflected by the different major metabolites formed in rat and pig liver microsomal incubations. Metabolites **6** and **7**a are the major metabolites formed, the first for pig and the second for rat liver microsomal incubation. MS data indicate the formation of only mono-oxygenated metabolites of tacrine. Detailed structural elucidation of the biological degradation products is described in Section 3.3.4.

Table 3.3 Chemical and bioactivity information of tacrine metabolites. Summary of biological degradation compounds formed after 1 h incubation with rat or pig liver microsomes. Their averaged retention time, their %RSD, the average corresponding bioactivities (when found) with %RSD and corresponding *m/z* values of molecular ions and their fragments are shown. Moreover the probable molecular formulas with error margin in ppm predicted by Agilent MassHunter Qualitative Analysis software are also shown. The bioactivity measured for compounds **7**, **7**b, **16**, **17** and **18** was around the detection limit and therefore not depicted in the table.

CYPs	Active peaks	RT average n=4	%RSD n=4	Activity average n=4	%RSD n=4	MS *m/z*	MS/MS *m/z*	Most likely molecular formula	Error (ppm)
Rat	**6**	10.30	1.28	1.3	11.3	215.1176	197.11;182.08	$C_{13}H_{15}N_2O^+$	1.4
	7	10.92	1.58	-	-	215.1176	197.11;182.08	$C_{13}H_{15}N_2O^+$	1.3
	7a	11.60	1.09	5.0	13.5	215.1176	197.11;182.08	$C_{13}H_{15}N_2O^+$	1.3
	7b	12.14	1.47	-	-	215.1174	197.11;182.08	$C_{13}H_{15}N_2O^+$	2.4
	15	19.86	0.51	12.6	4.9	199.1225	183.09;171.09	$C_{13}H_{15}N_2^+$	2.2
Pig	**6**	10.54	1.13	2.4	10.8	215.1173	197.11;182.08	$C_{13}H_{15}N_2O^+$	2.7
	7	10.96	0.68	-	-	215.1173	197.11;182.08	$C_{13}H_{15}N_2O^+$	2.8
	7a	11.88	0.97	1.0	7.6	215.1173	197.11;182.08	$C_{13}H_{15}N_2O^+$	2.6
	7b	-	-	-	-	-	-	-	-
	15	20.15	0.47	12.1	3.3	199.1229	182.08;171.09	$C_{13}H_{15}N_2^+$	0.5

3.3.4 Qualitative comparison between chemical and biological degradation

The results of this study showed that at least six mono-oxygenated compounds (*m/z* 215) were formed both by chemical degradation (Table 3.2) and by biological degradation (Table 3.3). This was judged by similarity in retention times, MS and MS/MS data. In Figure 3.6, extracted ion chromatograms of *m/z* 215 are shown for the chemical degradation with H_2O_2 (upper line) and the biological degradation with rat (bottom line) and pig (middle line) liver microsomes.

Fragmentation of the parent drug tacrine ($[M+H]^+$ with *m/z* 199) results in four major fragments (Figure 3.7A). Somewhat surprisingly, the loss of NH_3 to a fragment ion with *m/z* 182 is only a minor peak. The major fragment ion with *m/z* 171 can be explained by a retro-Diels-Alder fragmentation resulting in the loss of C_2H_4. The (unexpected) loss of CH_4 to an ion with *m/z* 183 may result in a ring closure with the NH_2 group. The two fragment ions with *m/z* 158 ($C_{10}H_{10}N_2^{+\bullet}$) and 144 ($C_{10}H_{10}N$) are also related to cleavages in the saturated ring of the molecule.

Also in Figure 3.7C, the MS/MS of peak **16** from pig liver microsomes is shown. Similar MS/MS spectra were obtained for peaks **16**, **17** and **18** in both samples (peak **18** for pig and

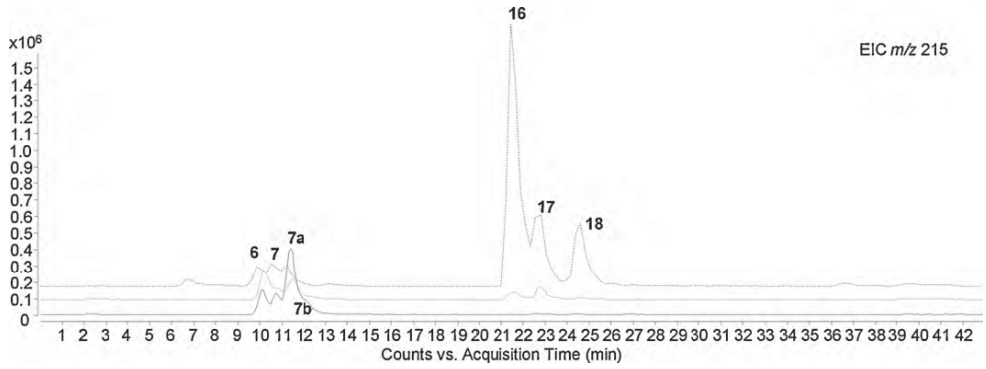


Figure 3.6 Comparison of mono-oxygenated tacrine metabolites with degradation products. The figure shows the EIC of *m/z* 215 (representing mono-oxygenated tacrine derivatives) for pig CYPs (middle line), rat CYPs (bottom line) and degradation products resulting from 3% hydrogen peroxide (upper line). The chemical and biological degradations showed the same mono-oxygenated compounds.

rat liver microsomes, due to its low intensity, did not show a good MS/MS spectrum), indicating that similar products were formed. All three compounds showed a protonated molecule with *m/z* 215 in MS and fragmentation in MS/MS identical to that of tacrine, but all with a shift of 16 Da in the *m/z* values. This fragmentation behavior indicates that for these three components oxidation took place in the aromatic ring. A fraction collected after the large scale chemical incubation which contained compounds **16**, **17** and **18** also indicated aromatic ring hydroxylations. As the retention times, MS and MS/MS data were similar for the chemical and the biological degradation peaks **16**, **17** and **18**, it was confirmed that the same three compounds were formed by both degradation experiments.

In the work of Pool *et al.* metabolic profiles of [^{14}C]-tacrine in plasma and urine from rat, dog and man were reported. From rat urine, only mono-hydroxylated metabolites were observed, as we also observed in our results. On the other hand, in dog and man urine, besides the mono-hydroxylated metabolites, di-hydroxylated metabolites were also found (Pool *et al.*, 1997). In our H_2O_2 degradation experiments, we also observed di- and tri-oxygenated products which might correspond to other di-and tri-hydroxylated metabolites and could in theory also be purified. Also by H_2O_2 degradation, ketones were formed and could be identified by MS and confirmed with NMR. In Figure 3.7D, the MS/MS spectrum of a degradation product with *m/z* 213 (peak **8** in Table 3.2) is shown. The ions with *m/z* 195, 183 and 171 correspond to the losses of water, $H_2C{=}O$, and $H_2C{=}C{=}O$, respectively. The *m/z* 185 corresponds to the loss of 28 Da. Elemental composition shows this corresponds to the loss of CO, and is not due the loss of C_2H_4 by a retro-Diels-Alder fragmentation. The ion with *m/z* 158 ($C_{10}H_{10}N_2^{+\bullet}$) is identical to one of the fragments of tacrine. The MS/MS data confirm the formation of a ketone at the non-aromatic ring.

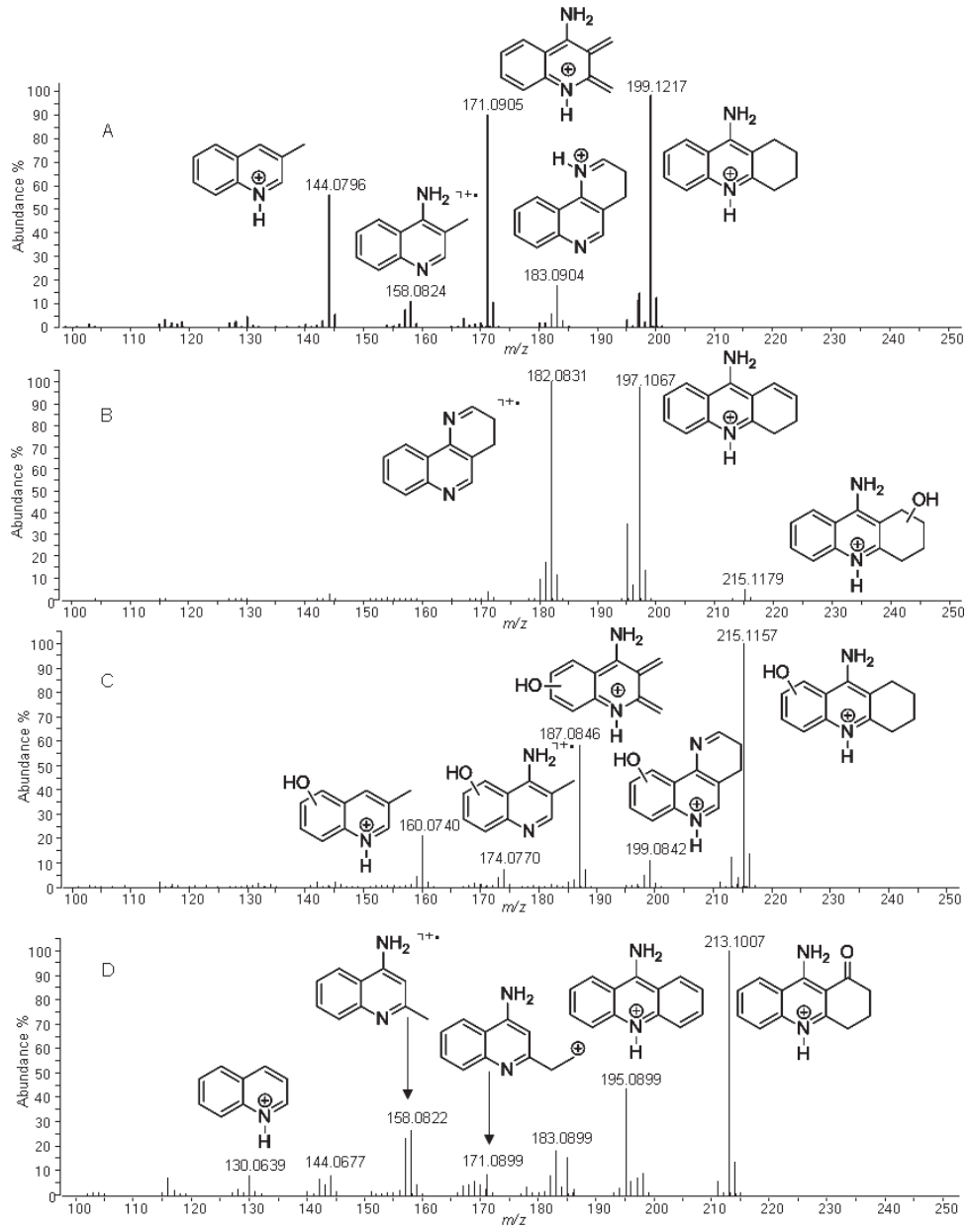


Figure 3.7 MS/MS spectra of tacrine and some of its oxidation products. (A) MS/MS of *m/z* 199 parent compound (tacrine), (B) MS/MS of *m/z* 215 corresponding to a derivative with a hydroxylation in the aliphatic ring, (C) MS/MS of *m/z* 215 corresponding to a derivative with a hydroxylation in the aromatic ring and (D) MS/MS of *m/z* 213 corresponding to an oxygenated derivative generated by chemical degradation with H_2O_2.

The NMR data led to the structural elucidation of the following compound: 9-amino-3,4-dihydroacridin-1(2H)-one, which confirms MS/MS data. Chemical shifts are referenced internally to solvent resonances (^{1}H: DMSO-d5, δ = 2.5 ppm, ^{13}C: DMSO-d6, 39.5 ppm). ^{1}H NMR (DMSO-d6, 500.23 MHz): δ = 8.70 (^{1}H, d, J = 8.4), 8.03–8.01 (^{2}H, m), 7.73 (^{1}H, m), 3.27 (^{2}H, t, J = 6.2), 2.73 (^{2}H, t, J = 6.5), 2.14 (^{2}H, quintet, J = 6.3). ^{13}C NMR (DMSO-d6, 125.78 MHz, δs from HMQC and HMBC spectra): δ = 199.58 (C), 162.38 (C), 157.77 (C), 137.55 (C), 135.60 (CH), 127.38 (CH), 125.14 (CH), 120.65 (CH), 117.36 (C), 105.03 (C), 38.70 (CH_2), 28.92 (CH_2), 20.44 (CH_2).

3.4 Conclusions

The present study showed a methodology using on-line AChE bioactivity profiling in parallel with HRMS for the bioactivity assessment and simultaneous identification of chemical and biological degradation products of tacrine. Chemical degradation was evaluated for the possibility to generate larger amounts of oxidative products also formed during metabolism. The study demonstrates bioactive products formed after both chemical and biological degradation (by CYP metabolism). In most chemical degradation studies, usually only the stability is investigated. This research showed that chemical degradation can yield similar compounds as biological degradation and also new bioactive compounds. Chemical degradation (H_2O_2 or UV in the case of tacrine) can thus conveniently be used to generate sufficient amounts of metabolites or other bioactive compounds also found after biological degradation for further analysis, e.g., NMR spectroscopy.

Acknowledgements

The work of the author Lygia Azevedo Marques was supported by the programme Alban, the European Union programme of high level scholarships for Latin America, scholarship (E06D-102010BR).

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