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Stability-indicating study of the anti-Alzheimer's drug galantamine hydrobromide

Lygia Azevedo Marques[a], Ismail Maada[a], Frans J.J. de Kanter[b], Henk Lingeman[a], Hubertus Irth[a], Wilfried M.A. Niessen[a], Martin Giera[a,*]

[a] VU University Amsterdam, Faculty of Sciences, BioMolecular Analysis Group, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands
[b] VU University Amsterdam, Faculty of Sciences, Division of Organic and Inorganic Chemistry, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands

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ABSTRACT

Galantamine hydrobromide was subjected to different stress conditions (acidic, alkaline, thermal, photolytic and oxidative). Degradation was found to occur under acidic, photolytic and oxidative conditions, while the drug was stable under alkaline and elevated temperature conditions. A stability-indicating reversed-phase liquid chromatographic method was developed for the determination of the drug in the presence of its degradation products. The method was validated for linearity, precision, accuracy, specificity, selectivity and intermediate precision. Additionally, the degradation kinetics of the drug was assessed in relevant cases. The kinetics followed a first order behavior in the case of acidic and photolytic degradation, while a two-phase kinetics behavior was found for the oxidative degradation. The degradation products were characterized by mass spectrometry and nuclear magnetic resonance spectroscopy. Dehydration, epimerization and *N*-oxidation were the main processes observed during the degradation of galantamine. Moreover, if sufficient material could be isolated the inhibitory activity against the target enzyme acetylcholinesterase was also assessed.

1. Introduction

Galantamine hydrobromide (**1**) is an acetylcholinesterase (AChE) inhibitor which is used to moderate and delay the manifestation of Alzheimer's disease (AD) symptoms. It is the AD drug most recently approved by the FDA [1]. **1** is a tertiary alkaloid, isolated from the bulbs and flowers of Caucasian snowdrop (*Galanthus woronowii*, Amaryllidaceae). The cost of obtaining galantamine from natural sources (*Narcissus* species) is very high [2]. A total synthesis procedure via 2-narwedine has been extensively worked out for large-scale industrial production [3]. **1** was launched under the commercial name of Reminyl® which was changed in 2005 to Razadyne® [4,5]. **1** is monographed by the European Pharmacopoeia and the United States Pharmacopoeia [6,7].

Although **1** is on the market for a couple of years, only a few studies about its stability have been described [4,8], in spite of the fact that stability testing is an important topic in order to ensure the quality and the safety of drugs [9].

Up to now, accelerated stability studies for two salts of galantamine: galantamine lactate and galantamine hydrobromide (**1**) have been described. The stability so far was investigated using different pH values and different temperatures [4]. A degradation study of **1** in Reminyl tablets also has been published. In this study, the main objective was to compare two different separation methodologies, namely: reversed-phase liquid chromatography with UV detection (RP-LC–UV) and capillary electrophoresis with tandem mass spectrometric detection (CE–MS/MS). The study was based on ICH guidelines, but only part of the mandatory stress conditions were investigated [8].

In the here presented work, a fully validated stability assessment for **1** is presented, including degradation kinetics, structure elucidation of the degradation products, and bioactivity assessment against the target enzyme AChE. The degradation products were separated using a RP-LC–UV method and spectroscopic and spectrometric methods were used for structural elucidation. After subsequent semi-preparative isolation, particular degradation products were tested for their inhibitory effect against the target enzyme AChE.

2. Experimental

2.1. Materials

All solvents used were of HPLC grade. Methanol (MeOH) and acetonitrile (ACN) were purchased from Biosolve (Valkenswaard, the Netherlands), Milli-Q water was obtained from a Milli-Q purifica-

* Corresponding author.
E-mail address: mgiera@few.vu.nl (M. Giera).

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tion system (Millipore, Amsterdam, The Netherlands). Ammonium acetate came from J.T. Baker B.V. (Deventer, The Netherlands). Galantamine hydrobromide (**1**) was supplied by Alexis (Lausen, Switzerland) and kindly supplied by Janssen-Cilag (Tilburg, The Netherlands). A stock solution of **1** (5 mM) was prepared in water and demonstrated to be stable for 5 months at 4 °C. Hydrogen peroxide 30% came from Merck (Darmstadt, Germany). For bioactivity measurements, the AChE (EC 3.1.1.7) type VI-S from *Electrophorus electricus* (electric eel) was obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands). 7-Acetoxy-1-methylquinolinium iodide (AMQI) was purchased from Fluka (Buchs, Switzerland). The enzyme and the substrate stock solutions were kept at −80 °C until use. 96-well black, solid plates were from Brand (Wertheim, Germany). All other chemicals were purchased from Sigma–Aldrich (Schnelldorf, Germany).

2.2. Instrumentation

2.2.1. Analytical liquid chromatography

RP-LC for analytical analysis was performed with a quaternary Agilent HPLC 1100 series pump (Agilent Technologies, Waldbronn, Germany), delivering a gradient of 10 mM ammonium acetate (pH 5.8): MeOH at 200 μl/min (solvent A: 10 mM ammonium acetate (pH 5.8): MeOH (95:5, v/v) and solvent B: 10 mM ammonium acetate (pH 5.8): MeOH (5:95, v/v). The gradient program was as follows: 0–5 min 0% B, followed by an increase to 90% B in 40 min, which was held for 5 min. The autosampler was a Gilson model 234 (Villiers Le Bel, France) with a 25 μl injection loop. Separation was performed on a Waters SunFire C_{18} (150 mm × 2.1 mm, 3.5 μm) (Waters, Milford, MA, USA) column used in combination with a Phenomenex C_{18} (4 mm × 2 mm, 5 μm) precolumn cartridge (Aschaffenburg, Germany). The temperature was kept at 30 °C by a Spark column oven (Spark Holland, Emmen, The Netherlands). The column effluent was monitored by UV absorption using an Agilent 1100 series diode-array detector (DAD). Kinetics data were evaluated at a wavelength of 290 nm.

2.2.2. Semi-preparative liquid chromatography

Semi-preparative RP-LC analysis was performed with a binary HPLC system consisting of two Shimadzu LC-10Ai pumps (Shimadzu, 's Hertogenbosch, The Netherlands), delivering the same solvents as used for the analytical separation at a flow rate of 4 ml/min. The injection was done using a Valco manual injection valve (Amstelveen, The Netherlands), with a 250 μl injection loop. The separation was performed on a Merck Purospher STAR RP-18e (250 mm × 10 mm, 5 μm) (Darmstadt, Germany), including a precolumn of the same material (10 mm × 4 mm). The temperature was held at 30 °C by a Spark column oven. For the collection of the degradation products under acidic conditions, the same gradient was used as for the analytical separation. The applied gradient for the preparative separation of the degradation products formed under irradiation conditions was as follows: 0 to 40 min 0% B to 20% B ramped to 80% B at 55 min. The column effluent was monitored both at 290 nm and 215 nm. All fractions were collected manually.

2.2.3. Light source

The Xenon Arc lamp used for the irradiation of **1** was from AEG (Nürnberg, Germany) and operated at 150 Watt.

2.2.4. Mass spectrometric analysis

For the MS analysis of oxidative degradation products in deuterated water, a model 6520 Q-TOF mass spectrometer (Agilent Technologies, Waldbronn, Germany) was used. Analyses were performed with electrospray ionization (ESI) in the positive-ion mode with direct infusion at a flow rate of 5 μl/min. The following conditions were used: interface voltage 3.5 kV, nebulizer and desolvation gas (Nitrogen 99.9990%) 15 psi and 4 l/min, respectively and a source temperature of 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 a collision energy of 30 eV. Argon (99.9995%) was used as collision gas. High resolution measurements were acquired after tuning and instrument calibration according to the manufacturer's specifications.

Degradation products formed under irradiation and acidic conditions were analyzed using a Shimadzu IT-TOF instrument equipped with an ESI source operated in positive-ion mode. The curved desolvation line and the heating block were set at 200 °C, the interface voltage was set at 4.5 kV, while a voltage of 1.7 kV was applied for the detector. Nitrogen (99.9990%) was used as a nebulizing gas at a flow of 1.5 l/min and as drying gas at a flow of 10 l/min. For the fragmentation experiments, argon (99.9995%) was used as collision gas.

In the full-spectrum mode, data were acquired from m/z 100–1000, with an ion accumulation time of 30 ms. MS^2 experiments were performed with an ion isolation time of 30 ms, an isolation width set at 3 m/z units, and a frequency (q) of 45 kHz. Both collision energy and gas were set at 50%. MS^3 parameters were the same as used for MS^2 measurements.

2.2.5. NMR analysis

All NMR measurements were performed on a Bruker Avance 500 (Fallanden, Switzerland), equipped with a cryoprobe, or a Bruker 400 (Fallanden, Switzerland); the residual methanol-d4 signal was used as reference. ^{1}H NMR measurements were carried out at 500 or 400 MHz, while the ^{13}C NMR experiments were performed at 100 or 125 MHz.

2.3. Protocols

2.3.1. Method validation

Validation of the optimized RP-LC system was carried out according to ICH guidelines [10] with respect to the following parameters: linearity, precision, accuracy and specificity. Additionally, system suitability parameters were evaluated.

2.3.2. Application of the validated RP-LC method for the determination of galantamine (*1*) in Reminyl retard capsules

The validated RP-LC method was applied to quantify the amount of **1** in Reminyl retard capsules. An accurately weighted aliquot was taken in duplicate from a total sample of 10 homogenized Reminyl capsules. The sample was diluted to an expected concentration of 1.7 mM with MeOH and submitted to ultrasonication for 30 min. The solution was filtered through a 0.45 μm filter and subsequently diluted with eluent A to an expected, concentration of 30 μM. This solution was analyzed in triplicate with the above described analytical RP-LC system. From the results, the capsule content was determined.

2.4. Degradation and kinetic studies

A stock solution of **1** (5 mM) in water was prepared and stored at 4 °C until use. This stock solution was appropriately diluted for all degradation and kinetic experiments to reach a final test concentration of 200 μM. The degradation and kinetics of **1** were investigated under acidic, basic, oxidative and elevated temperature conditions as well as under irradiation with a Xenon Arc lamp at 150 W. Samples were placed in the autosampler tray just before injection. The kinetics was determined for all conditions under which degradation was observed. Data were processed using the software Prism version 5.08 (Graph Pad, San Diego, CA, USA).

2.4.1. Acidic conditions

1 was treated with 1 M hydrochloric acid (HCl) at 80 °C. The starting concentration (c_0) was measured by preheating the solution of 1 M HCl to 80 °C and quickly removing an aliquot of 200 μl after the stock solution of **1** has been added. The aliquot (200 μl) was cooled to room temperature (RT) and 100 μl was taken and diluted with 210 μl of cold mobile phase A and 90 μl of 1 M NaOH for neutralization. This resulted in a final concentration of 50 μM of **1** at time 0 min. From 0 to 20 min, an aliquot was drawn every 5 min, from 20 until 120 min, aliquots were collected in 10 min intervals. Finally, from 120 until 300 min, aliquots were collected in 30 min intervals

2.4.2. Alkaline conditions

1 was heated to 80 °C in a 1 M solution of sodium hydroxide (NaOH). The same sampling procedure as described for the acidic conditions was applied, except that 1 M HCl was added after sampling instead of 1 M NaOH (in Section 2.4.1)

2.4.3. Oxidative conditions

1 was heated to 80 °C in 3% H_2O_2. The same sampling procedure as described for the acidic and alkaline conditions was applied, except that 300 μl of cold mobile phase A was added instead of 210 μl plus 90 μl 1 M NaOH. The final aliquot was taken at 240 min after starting the collection process. As the oxidative degradation of **1** was a rapid process, the storage stability at 4 °C was studied and the samples proved to be stable under these conditions for at least 2 days. For this reason, samples could be stored in the fridge at 4 °C until injection

2.4.4. Elevated temperatures

As control experiment, for the above mentioned investigations, **1** was heated to 80 °C in water for 300 min as well as for 2 months inside a column oven. The same sampling procedure as described for the oxidative conditions was applied (addition of 300 μl of cold mobile phase A to the sample). For long term stability testing, sampling was performed at the following intervals: from the starting point until 7 days, one sample per day was taken. From day 7 until day 15, one sample was taken and from day 15 until day 60 samples were taken every 15 days. As a control, a solution of **1** wrapped in aluminum foil, stored at room temperature was treated in the same manner.

2.4.5. Irradiation

A solution of **1** in water was placed in a Duran® glass flask and irradiated with a Xenon Arc lamp at 150 W. Duran® glass has a "natural" cut off at 310 nm according to the manufacturer's specifications. In order to exclude possible effects of heat due to the irradiation, a control sample in an aluminum foil covered flask was irradiated. The same sampling procedure as described for the oxidative conditions was applied (addition of 300 μl of cold mobile phase A to the sample with a final aliquot taken at 180 min after initialization).

2.5. Large scale preparation of degradation products

2.5.1. Degradation under acidic conditions

For the large scale generation of degradation products, two separate reaction batches of 6 mg of **1** in 10 ml of 1 M HCl were refluxed for 2 h. The solutions were alkalized with 3 ml of an aqueous sodium hydroxide solution (5 M) and extracted with three portions of 5 ml dichloromethane (DCM). The combined organic layers were washed carefully with 20 ml of water. The organic phase was dried over anhydrous magnesium sulfate, filtered and evaporated to dryness under a gentle stream of nitrogen. The resulting residue was dissolved in 1 ml of mobile phase A and 250 μl portions were injected into the specified preparative LC system. The collected fractions were pooled and the DCM extraction was repeated, using 3 portions of 20 ml DCM. The solid residues were dried under vacuum, weighted and submitted to NMR analysis and/or bioaffinity testing.

2.5.2. Oxidative degradation products

For evaluation of the major degradation product under oxidative conditions, 2.5 mg of **1** was incubated in 3% H_2O_2, which was prepared from 30% H_2O_2 solution by dilution with deuterium oxide (D_2O), or water (H_2O) in the case of the control experiment. The total volume of this solution was 5 ml. The solution was refluxed for 2 h at 80 °C. The resulting solution was diluted with D_2O and directly infused into a mass spectrometer, as described in Section 2.2.4.

2.5.3. Photolytic degradation products

For the generation of photolytic degradation products, three separate batches of 4 mg of **1** in 10 ml of water were irradiated under the above stated conditions. The liquid–liquid extraction and preparative isolation were applied as described in Section 2.5.1. Addition of 200 μl of 5 M NaOH was used to alkalify the solution. The solid residues were dried under vacuum, weighted and submitted to NMR analysis. Each collected fraction was additionally injected into the analytical LC system to control the purity.

2.6. Acetylcholinesterase inhibition assay

The AChE activity was determined according to [11] with some modifications: 10 mM stock solutions in MeOH of the isolated degradation products, formed under acidic conditions, were prepared from the purified material and used for further dilutions. The AChE bioactivity assay was measured in a 96-well plate using AChE dissolved in 20 mM Tris–HCl buffer pH 7.5, 2 g/l PEG 6000 and 1 g/l ELISA blocking reagent. As substrate, AMQI 2 μM in 50 mM of citrate buffer at pH 5 was used. 8 μl of a solution containing the degradation products (10 mM, 0.2 mM) in MeOH were dried in the wells. First, 100 μl of the enzyme stock solution (0.1 U/ml) was added to all wells of the plate with a multichannel pipette. 100 μl of enzyme solution was added to the wells containing the test compounds (dried). This resulted in the stock solution of the analytes. From these wells, 100 μl was taken and subsequently diluted 1:1 into the other wells already containing 100 μl enzyme solution. The dilutions on the microtiterplate were performed on ice. After the plate was heated to RT, 100 μl of the substrate solution was added to all wells. The final concentrations for the enzyme and substrate were 0.05 U/ml and 1 μM, respectively. The fluorescence intensity was measured 30 s after substrate addition for 6 time points within 5 min using a Victor^3 plate reader (PerkinElmer, Waltham, MA, USA) at 405 nm and 520 nm for excitation and emission. From this data, the slope of the enzymatic reaction was generated and plotted against the decimal logarithm of the concentration of the substance added. As a positive control, the IC_{50} of **1** using a stock solution of 10 mM was measured following the same procedure as described above. The IC_{50} curves were generated with the software Prism version 5.08. (Graph Pad, San Diego, CA, USA).

3. Results and discussion

3.1. Validation of the RP-LC method

The following system suitability parameters were found: capacity factor 6.5, theoretical plate number 41636 and a tailing factor of 1.3. The results are in accordance with the USP method for **1**, specifying a tailing factor not higher than 2.0 [7]. The response of **1** was linear (R^2 0.9990) in the working range between 2.5 and 60 μM

Table 1
Recovery studies ($n = 3$) performed under acidic and basic conditions.

Actual concentration (μM)	Calculated concentration (μM)	RSD (%)	Recovery (%)
Acid			
5.0	5.1	1.2	102.3
10.0	9.9	0.7	98.9
15.0	14.9	1.4	99.4
30.0	30.3	0.6	100.9
Base			
5.0	5.1	0.9	102.5
10.0	10.2	1.4	101.2
15.0	15.1	2.1	100.9
30.0	30.6	0.5	101.9

($y = 25.63(\pm 0.10)x - 7.35(\pm 2.90)$). Repeatability and intermediate precision (%RSD) was less than 2%. A one-way ANOVA proved that there is no significant difference between days. Good accuracy with quantitative recoveries was achieved, indicating that the method enables accurate determination of the analytes (Table 1).

The accuracy in the case of the oxidative stress conditions could not be evaluated, due to the fast degradation of **1.** The irradiation experiments and elevated temperature were carried out in water, meaning that no matrix effects were to be expected. Specificity was assessed by applying the peak purity function of the Chemstation software to all samples under all stability testing conditions. The wavelength 290 nm was found to be more specific and therefore, used for the determination of **1**.

3.1.1. Application of the validated RP-LC method for determination of galantamine in Reminyl® retard capsules

The validated RP-LC method was applied to the quantification of **1** in Reminyl® retard capsules. The mean recovery obtained was $100 \pm 1.5\%$ ($n = 6$). The result obtained is in accordance with the USP, which demands a value between 98 and 102%.

3.2. Degradation and kinetic studies

Degradation studies provide information on the quality of a drug under the influence of different conditions and the degradation kinetics allow measurement of the speed of these processes. This is important to verify the stability of the drug. **1** proved to be stable for at least 300 min at 80 °C under alkaline conditions and for at least 60 days under neutral conditions. However, when **1** was exposed to a strong light source, acidic conditions or oxidative conditions, degradation occurred. Table 2 gives an overview of the found degradation products classified according to the various stability conditions under which they are formed. Fig. 1 shows the chromatograms at 215 nm of the separation of the degradation products under acidic, oxidative and photolytic conditions.

3.2.1. Acidic conditions

A solution of **1** was degraded at 80 °C in 1 M HCl. The semi-logarithmic plot of the concentration of **1** (μM) against time, indicated an apparent first order degradation kinetics. The degradation rate constant (k) was calculated by the use of the equation $\ln c = \ln c_0 - kt$, where c_0 is the concentration of **1** at time $t = 0$ and c is the concentration at time t. Using the described experimental conditions, the regression coefficient (R^2) for the logarithmic plot was determined to be 0.9909, while k was $0.2185 \pm 0.005242\ h^{-1}$. In acidic media at 80 °C after 300 min, one major and four minor compounds (below 1.7%) were formed. After 120 min under reflux, three major and two minor compounds were formed. The three major compounds (**2**, **3** and **4**) could be collected, two of which could be identified by NMR (see Table 2).

3.2.2. Oxidative conditions

A solution of **1** was treated at 80 °C with 3% H_2O_2. The semi-logarithmic plot of the concentration of **1** (μM) against time indicated an apparent two-phase kinetics. The degradation rate constants (k) were: k fast: $5.162 \pm 0.6595\ h^{-1}$ and k slow: $0.7848 \pm 0.09513\ h^{-1}$ with $40.63 \pm 3.99\%$ of fast decay. The regression coefficient (R^2) for the plot was determined to be 0.9992. A first hypothesis for this behavior was the degradation of H_2O_2 during time. To confirm this hypothesis, H_2O_2 was titrated with potassium permanganate ($KMnO_4$) in acidic media [12]. The concentrations found before and after heating were not significantly different, meaning that the observed kinetics is not related to the degradation of H_2O_2 over time. Hence, an explanation of the apparent two-phase kinetic behavior cannot be given. The degradation at 80 °C and after 2 h under reflux revealed the production of one major compound and numerous unidentifiable products at rather low amounts (<0.5%). The major compound was identified as **5** by MS using the methodology described in Section 2.5.2.

3.2.3. Photolytic conditions

A solution of **1** was irradiated with a Xenon Arc lamp. The semi-logarithmic plot of the concentration of **1** (μM) against the time indicated an apparent first order degradation behavior. Under the described experimental conditions, the regression coefficient (R^2) for the logarithmic plot was determined to be 0.9976, while k was $0.7453 \pm 0.01023\ h^{-1}$. A control sample covered with aluminum foil was submitted to the same conditions; the analysis revealed that no degradation occurred.

3.3. Structural elucidation of degradation products

For all detected degradation products, MS^n (with up to $n = 3$) spectral data were acquired. The m/z values as well as the predicted molecular formulas are given in Supplementary material S1. Unfortunately, a structural elucidation solely based on MS was not possible for most of the substances, because there were no distinctive fragments to allow discrimination between the various isomeric structures formed. This was especially true for the series of isomeric substances formed under photolytic conditions. However, most of the fragments found were in good agreement with previously published data [13–15]. Due to this, NMR spectroscopy was additionally employed wherever possible to elucidate the structures of the formed degradation products.

3.3.1. Degradation under acidic conditions

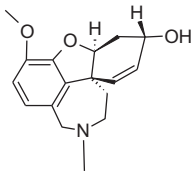
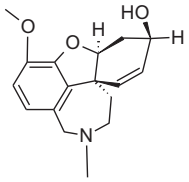
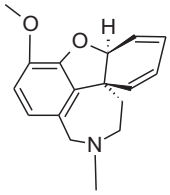
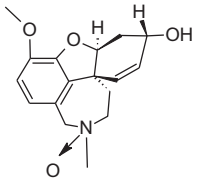
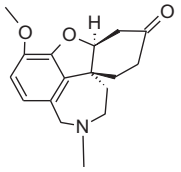
After the initial detection of the degradation products under acidic conditions, the compounds were isolated for a complete structure elucidation as described in Section 2.5. The yield for compounds **2**, **3** and **4** (numbers according to Table 2) was 2.9, 1.8 and 1.7 mg, respectively. This material was used for all further spectroscopic analyses.

NMR data was conclusive to identify peak **2** as epigalantamine and peak **4** as 3-methoxy-11-methyl-9,10,11,12-tetrahydro-4aH-benzo[2,3] benzofuro[4,3-cd]azepine referring to earlier published ^1H NMR data [14,15]. Both structures can be expected under acidic conditions as epimerization and dehydration are well known processes under such conditions [16]. The recorded MS and MS^n data can be found in Supplementary material S1.

3.3.2. Oxidative degradation products

The chromatogram of **1** heated in a solution of 3% H_2O_2 showed one major degradation product, besides a large number of minor degradation products. In this study, the focus was on the extraction of the major component. Unfortunately, it was not possible to extract the formed degradation product into a variety of organic

Table 2
Summary of analytical data. All detected substances are shown together with their corresponding retention time (R_t), m/z of the protonated molecule, most likely molecular formula and UV maximum. ^{1}H NMR references and structures are given in cases where substances were identified based on spectroscopic data including NMR.

Number	R_t (min)	Test condition	m/z (error – ppm)[a]	Most likely molecular formula	^{1}H NMR (ref)	UV max[b] (nm)	Structure
1 (base form)	16.3	Parent compound	288.1593 (0.42)	$C_{17}H_{22}NO_3^+$	[13]	215	 Galantamine
2	18.4	Acidic	288.1593 (0.42)	$C_{17}H_{22}NO_3^+$	[14]	215	 Epigalantamine
3	22.6	Acidic	270.1487 (0.58)	$C_{17}H_{20}NO_2^+$	–	210	Not determined
4	24.3	Acidic	270.1480 (0.58)	$C_{17}H_{20}NO_2^+$	[15]	210	 3-Methoxy-11-methyl-9,10,11,12-tetrahydro-4aH-benzo[2,3] benzofuro[4,3-cd]azepine
5	17.8	Oxidative	304.1551 (−2.5)	$C_{17}H_{22}NO_4^+$	–	215	 Galantamine-*N*-oxide
6	12.8	Photolytic	306.1680 (6.5)	$C_{17}H_{24}NO_4^+$	–	210	Not determined
7	14.3	Photolytic	288.1573 (7.4)	$C_{17}H_{22}NO_3^+$	–	210	Not determined
8	15.0	Photolytic	306.1680 (6.5)	$C_{17}H_{24}NO_4^+$	–	210	Not determined
9	17.5	Photolytic	288.1572 (7.7)	$C_{17}H_{22}NO_3^+$	[21]	210	 3-Methoxy-11-methyl-7,8,9,10,11,12-hexahydro-4aH-benzo[2,3] benzofuro[4,3-cd] azepin-6(5H)-one
2	18.0	Photolytic	288.1577 (5.9)	$C_{17}H_{22}NO_3^+$	[14]	215	See **2**
10	19.0	Photolytic	288.1577 (5.9)	$C_{17}H_{22}NO_3^+$	–	210	Not determined
11	26.3	Photolytic	288.1571 (8.1)	$C_{17}H_{22}NO_3^+$	–	210	Not determined

[a] Error given in parts per million with respect to the calculated molecular formula.
[b] UV spectra for all degradation products can be found in Supplementary material S2.

solvents (e.g. DCM, ethyl acetate, ethyl acetate containing 0.05 M triethylamine (TEA) (0.7%, v/v) and methyl-*tert*-butyl ether).

As the isolation of the substance, suspected to be Galantamine *N*-oxide (**5**) (number according to Table 2) was unsuccessful, an MS based identification strategy was applied as described in Section 2.5.2. Earlier studies dealing with the extraction of diphenhydramine *N*-oxide [17] reported this type of problems, which the authors related to the extreme hydrophilicity of *N*-oxides and adhesion to glassware. In the article cited, the recoveries were found to be satisfactory by extracting the diphenhydramine *N*-oxide with ethyl acetate containing 0.72% (v/v) of TEA. Unfortunately, in the present study this solvent system did not allow the extraction of solute **5**. Another study related to the phase I metabolism of **1** claimed the MS identification of **5** [18]. Although our MS data were in good agreement with earlier published data [18] (the recorded MS and MS^n data can be found in Supplementary material S1),

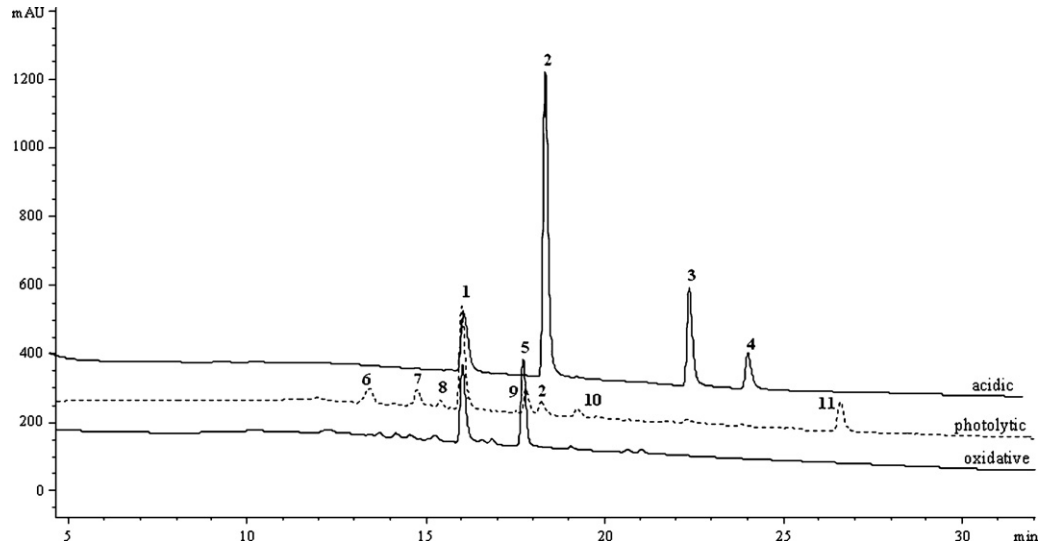


Fig. 1. RPLC chromatograms (215 nm) of degradation studies performed under acidic, photolytic and oxidative conditions. Galantamine corresponds to peak **1** in the chromatogram, other peak numbers refer to Table 2.

the lack of additional NMR data, or other proof for the definitive identity of **5** required another strategy proving the *N*-oxides identity. The necessity of doing so was additionally based on the fact that, although the main reaction of H_2O_2 with amine-containing substances is the formation of *N*-oxides, H_2O_2 can also cause the hydroxylation at double bonds or aromatic systems [19]. In both cases, an *m*/*z* value of +16 would be found for the molecule, thereby rendering a solely MS based distinction between hydroxylation and *N*-oxide formation impossible. Therefore, hydrogen/deuterium exchange experiments were performed [20]. In this case, the solu-

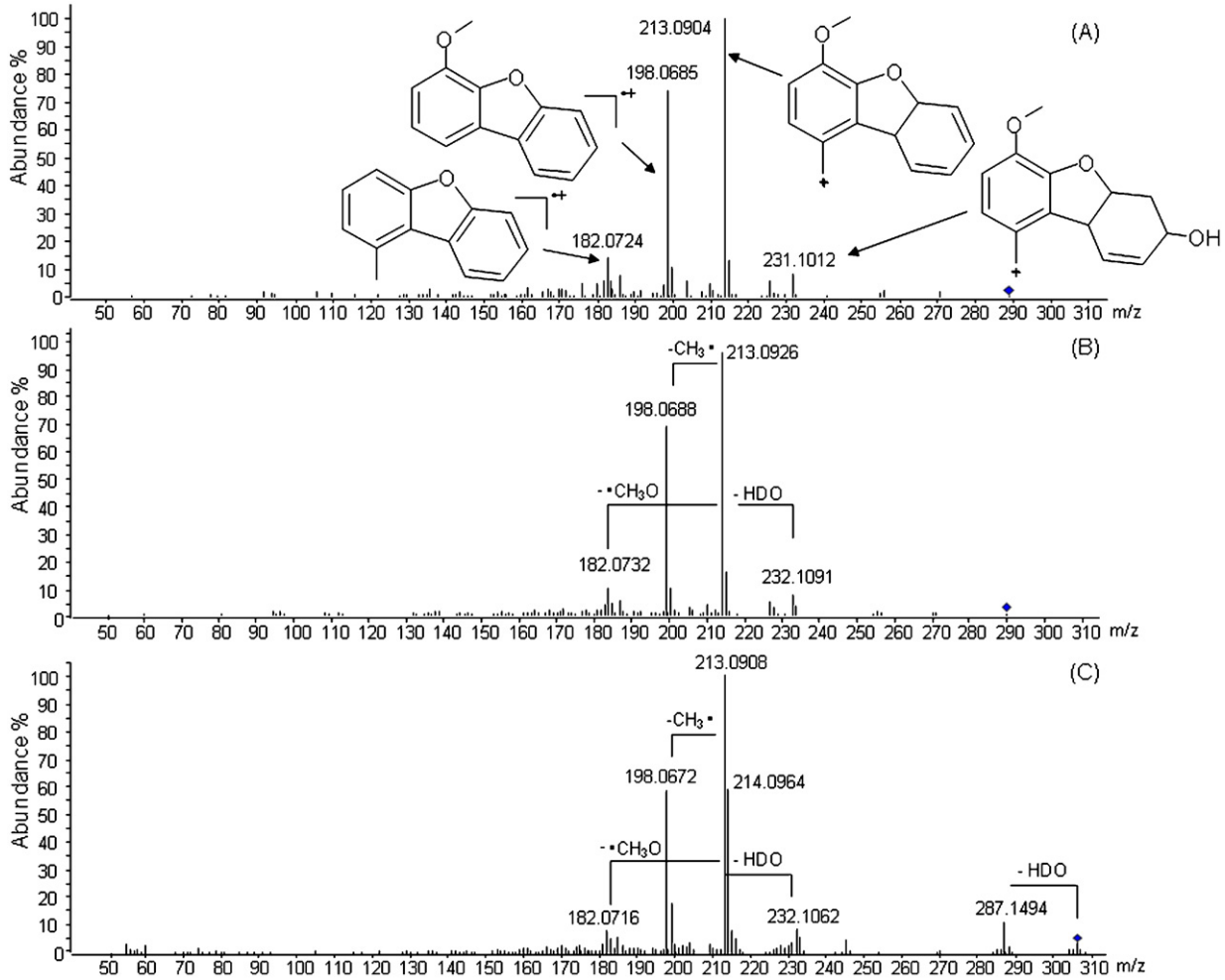


Fig. 2. MS/MS spectra of non deuterated galantamine (A), deuterated galantamine (B) and deuterated *N*-oxide-galantamine (C). For details, refer to the text.

tion of **1,** after oxidative degradation, was diluted with D_2O before infusion into the MS. Subsequently, the theoretical number of exchanged hydrogen atoms was compared to the found values. For **1** itself, an *m*/*z* increase of +2 is observed when comparing the spectra in the deuterated with the non-deuterated solvent. This is due to hydrogen exchange of the hydroxyl hydrogen atom and protonation by D^+ rather than H^+. Relative to **1**, the oxidative degradation product would show an *m*/*z* increase of +19 in the deuterated solvent if an additional hydroxyl function is introduced and an *m*/*z* increase of +18 in the case of an *N*-oxide. An *m*/*z* increase after the treatment of **1** with H_2O_2 of +18 was observed, thereby clearly indicating the formation of an *N*-oxide. This assumption could be further strengthened by the acquisition of MS/MS data (Fig. 2), which clearly showed the formation of an identical fragment after the cleavage of the azepin ring and a water loss of the molecules, when comparing **5** with **1**.

3.3.3. Photolytic degradation products

Due to the large number of products formed by the photolytic degradation, a large scale preparation and isolation of these products was not feasible for all of them. The recorded MS and MS^n data can be found in Supplementary material S1. The three major compounds were isolated with the preparative RP-LC system described above. For all three isolated substances, NMR measurements were carried out, as specified in Table 2 [21]. Analytical RP-LC–UV analysis revealed that the purity of the collected fractions was lower than 70%. This and the fact that the overall recovery achieved, for each peak, was less than 10% (with respect to the total amount of **1**), rendered a full structural elucidation impossible. A better chromatographic separation of the substances in the preparative system could unfortunately not be achieved by exchanging MeOH for ACN in the eluents. However, an improved analytical separation resulted in a cleaner UV trace and also MS^n measurements of the major photolytic degradation compounds, indicating a number of isomers of **1** were formed.

3.4. Acetylcholinesterase inhibition assay

The AChE inhibition assay was only performed with the degradation products formed under acidic conditions (**2**, **3** and **4**). The purity of the substances was determined to be at least 95% according to analysis with the analytical LC-UV (215 nm) system. Based on the methodology described in Section 2.6, the IC_{50} value observed for **1** was 1.03 μM, which is in good agreement with previously published data (0.5–1.4 μM) [22,23]. The IC_{50} values for **2** and **4** were 425 and 692 μM, respectively. For substance **2**, this result was comparable with literature data [24]. For substance **4**, no comparative data were found in the literature. Substance **3** did not show any activity against AChE. Overall, the isolated degradation products showed a lower bioactivity compared to **1** itself.

4. Conclusions

A method to evaluate the stability of galantamine hydrobromide under different stress conditions was developed and validated. Galantamine proved to be stable under alkaline conditions and at elevated temperatures. On the other hand, under acidic, oxidative and photolytic conditions significant degradation occurred. All formed degradation products were characterized chemically and biochemically as far as possible. Moreover the degradation kinetics were also assessed and showed to follow first order kinetics for acidic and photolytic conditions. However, under oxidative conditions, a two-phase kinetics behavior was observed, for which no valid explanation can be given. All tested degradation products of **1** showed very weak inhibitory activities against the target enzyme AChE.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2011.01.022.

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