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

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Chapter 2

Analysis of acetylcholinesterase inhibitors: bioanalysis, degradation and metabolism

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Abstract

Alzheimer's is a neurodegenerative disease. Its symptoms are attributed to a deficiency of cholinergic neurotransmission. The drugs of choice for the treatment of Alzheimer's disease are acetylcholinesterase (AChE) inhibitors. Starting in the 1980's from non-specific AChE inhibitors, the first generation drugs such as physostigmine, a second generation of more selective and better tolerated products has been developed. Methods to detect and quantify these drugs and their metabolites in biological samples have been developed for analysis in plasma, blood, urine and cerebrospinal fluid. Diverse detection techniques have been used, such as ultraviolet, fluorescence, electrochemical and mass spectrometry. In this review, the methods applied to the analysis of these drugs and their metabolites in different biological matrices are reviewed and discussed. The stability of these drugs in biological matrices and under stress-conditions is also included in the discussion.

Introduction

Alzheimer's disease (AD) is an incurable, neurodegenerative, and terminal disease first described by the German neuropathologist Alois Alzheimer in 1906. In 2006, there were 26.6 million people suffering from it worldwide, mainly among people of over 65 years old. It is estimated that in the US annually ~$ 148 billion is spent in direct and indirect costs for Alzheimer and other dementias (Maslow, 2008). There are three major hypotheses to explain the cause of the disease: reduced availability of the neurotransmitter acetylcholine, deposition of the amyloid-β peptides, and abnormalities of the tau protein (Gauthier and Poirier, 2008). Oxidative stress is also believed to be a significant cause in the formation of the pathology (Mamelak, 2007). Given the increasing number of people suffering from AD, many studies are in progress on how to alter the course of the disease and how to improve the quality of life for people having dementia. A number of these studies are based on the hypothesis that AD is related to a reduced availability of the neurotransmitter acetylcholine, which may be caused by an increased activity of the enzyme acetylcholinesterase (AChE), which catalyzes the breakdown of acetylcholine into choline and acetic acid (Sugimoto, 2008). Acetylcholine is an important neurotransmitter in both the peripheral nervous system (PNS) and the central nervous system (CNS). Some of the drugs used in the treatment of AD are based on the inhibition of AChE, thereby increasing the level of available acetylcholine in the synaptic gap, in that way improving neuronal functions. This review pays attention to various analytical aspects related to (the development of) AChE inhibiting drugs currently applied in the treatment of AD, being: physostigmine (**1**), tacrine (**2**), donepezil (**3**), rivastigmine (**4**), galantamine (**5**), and huperzine A (**6**). The development of validated bioanalytical methods for the analysis of AChE inhibitors in biological samples is important in assessing the bioavailability and pharmacokinetics of these drugs as well as in investigations that lead to potential new drug candidates. In addition, methods described for the analysis of related compounds, like degradation products and metabolites are described in this review. This involves methods for stability indicating assays according to International Conference on Harmonization (ICH) guidelines, (ICH, 1995, ICH, 1996, ICH, 1999, ICH, 2003) which in many countries are used in regulatory affairs related to the registration of new drugs (Bakshi and Singh, 2002).

2.1 Physostigmine

Physostigmine (**1**), also called eserine, is an alkaloid found in Calabar beans, which are the seeds of the fabaceous plant Physostigma venenosum and which are poisonous to humans. This West African plant has been used as an ordeal poison in trials for witchcraft in Africa (Dworacek and Ruprecht, 2002). **1** can also be synthesized in several ways (Kawahara *et al.*, 2000, Mukai *et al.*, 2006, Nakagawa and Kawahara, 2000, Trost and Zhang, 2006). The structure of **1** is shown in Figure 2.1. Physostigmine (**1**) is normally used to treat glaucoma; moreover, it is an atropine antidote. Because **1** also is a reversible AChE inhibitor, capable to pass the

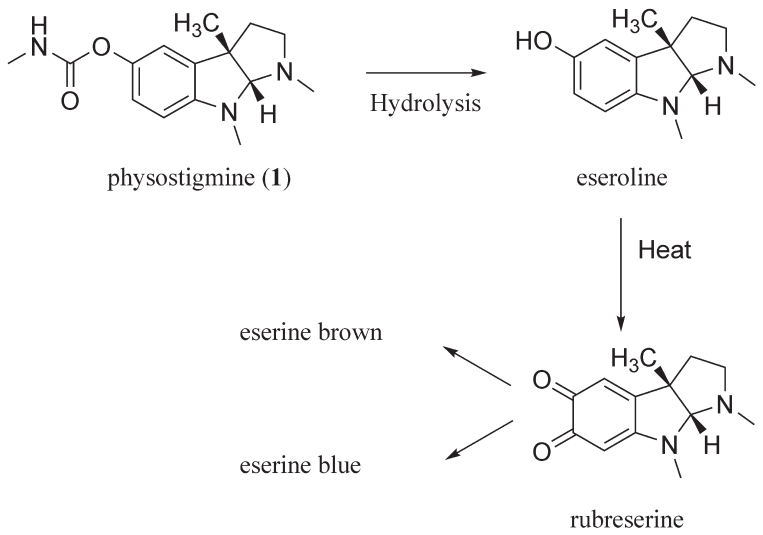


Figure 2.1 Decomposition pathway of physostigmine (**1**), (adapted with permission from Rubnov *et al.*, 1999).

blood-brain barrier, it had been tested for AD treatment. In initial studies with small groups of AD patients, the drug was effective to some degree in a few studies, while negative results were obtained in other studies. These ambiguous results are mainly due to the short half-life of **1** (15–30 min), and presumably to peripheral side effects (Sugimoto *et al.*, 2002).

In AD treatment, physostigmine salicylate has mostly been used. Due to side effects even in a controlled release formulation designed to overcome the short half-life, further research into this drug is no longer recommended (Coelho Filho and Birks, 2001). Details for the most relevant methods developed for the analysis of **1** are given in Table 2.1.

2.1.1 Degradation studies

Under alkaline conditions (pH>9), **1** is rapidly hydrolyzed to eseroline, in the same way it is hydrolyzed by the action of the AChE and by other esterases in plasma (Lawrence and Yatim, 1990). At pH>6, eseroline can undergo subsequent autoxidation to rubreserine. The pathway of the conversion of **1** into its degradation products is shown in Figure 2.1. Rubnov *et al.* developed an isocratic reversed-phase liquid chromatography (RPLC) method for the analysis of physostigmine salicylate and its degradation products (Rubnov *et al.*, 1999). RPLC on a C_{18} column was used with a mobile phase consisting of 50% acetonitrile (ACN) : 50% 0.1 M ammonium acetate buffer (pH 6.0). UV detection at 248 and 305 nm was used; 305 nm is the optimum wavelength for detection of degradation products, whereas for **1** a better sensitivity was achieved at 248 nm. The method was capable to follow the degradation of physostigmine salicylate to eseroline, rubreserine and (probably) eserine brown (Poobrasert and Cordell, 1997, Rubnov *et al.*, 1999). Another known degradation product of **1** is eserine blue, whose formula

was suggested by Salway in 1912 (Salway, 1912), but only in 1967 its structure, was proposed (Auterhoff and Hamacher, 1967). Poobrasert and Cordell found two additional blue degradation products of eserine, which were identified on basis of their NMR data (Poobrasert and Cordell, 1997). Overall, a final prove (NMR, crystal structure) for the structure of eserine blue could not be found in literature, which probably is why Rubnov *et al.* correctly suggested that further work is required to confirm the peak identities (Rubnov *et al.*, 1999).

2.1.2 Metabolism studies

Isaksson and Kissinger studied the metabolism of **1** in mouse liver microsomal incubations by liquid chromatography with dual-electrode amperometric detection. Proteins were precipitated with methanol and perchloric acid. **1** and its metabolites were separated by using a Biophase octyl column (250 × 4.6 mm, 5 µm) and a mobile phase consisting of 40% ACN : 60% 100 mM phosphate buffer pH 3 : 0.5% 17 mM sodium dodecylsulphate (SDS). They observed two main and six minor metabolites, but structure identification was not performed (Isaksson and Kissinger, 1987). From the work of Zhao *et al.*, it is known that eseroline (see Figure 2.1) is a metabolite of **1** (Zhao *et al.*, 2003).

2.1.3 Bioanalytical studies

The analysis of **1** in plasma and other biological fluids has been reported by various groups, mainly in the context of pharmacokinetic studies. Whelpton and Moore reported the development of a method for the quantification of **1** in plasma, whole blood and urine using electrochemical detection (ECD) with a dual-electrode system (Whelpton and Moore, 1985). Liquid-liquid extraction (LLE) of **1** from alkalinized blood and plasma was performed using diethyl ether, whereas for urine samples extraction with benzene was applied. The analysis of **1** in blood or plasma is hindered by the hydrolysis of the compound by plasma esterases in the sample tube. The addition of an excess of neostigmine to the biological samples after collection helped solving this problem (stabilizing agent). Better signals in ECD were obtained with alkaline buffer (pH 8.9). Separation was achieved by RPLC. The limit of detection (LOD) of the described method was 50 pg/mL in urine and 100 pg/mL in whole blood or plasma, using a total amount of **2** mL of sample and 25-50 pg/mL in plasma using 4 mL of sample (Whelpton and Moore, 1985). It was observed that the use of benzene for the extraction of urine gave less interfering peaks (Note: benzene is carcinogenic and should be replaced wherever possible by toluene; it is not known if toluene has the same extraction efficiency as benzene).

Somani and Khalique developed a method for determination of **1** in rat plasma and brain. Plasma and brain were first precipitated with trichlororacetic acid (TCA) and then 1- naphthlmethylcarbamate (carbaryl) was added. This was followed by chloroform extraction. **1** and its hydrolyzed product eseroline were separated by RPLC. The eluted compounds were detected using UV detection at 245 nm. The analytical recoveries were higher than 95% (Somani and Khalique, 1985).

Brodie *et al.* applied fluorescence detection (excitation and emission wavelength of 254 and

346 nm, respectively) for the determination of **1** in plasma (Brodie *et al.*, 1987). After mixing the plasma with ammonium hydroxide, LLE was performed with methyl tert-butyl ether. Pyridostigmine was added as stabilizing agent. A LOD of 0.1 ng/mL was achieved also using 2 mL of blood sample (Brodie *et al.*, 1987).

Elsayed *et al.* reported a method involving ion-pair LLE, normal phase high performance liquid chromatography (HPLC) separation, and fluorescence detection (excitation and emission wavelength of 240 nm and 360 nm, respectively) for **1** in human plasma (Elsayed *et al.*, 1989). The analytes were first extracted from plasma with dichloromethane and then back-extracted into water containing 1 mM tetrabutylammonium. A silica column was used with a mobile phase of 20% ACN : 80% 0.01 M NaH_2PO_4 and 2.5 mM tetramethylammonium (pH 3.0). The LOD of the method was 0.1 ng/mL using 1 mL of a plasma sample (Elsayed *et al.*, 1989).

Unni *et al.* determined **1** in human plasma and cerebrospinal fluid (CSF) using liquid chromatography with electrochemical detection (Unni *et al.*, 1989). Compound **1** was extracted with diethylether from plasma samples (2 mL) after alkalization with 0.5 mL of an ammonium hydroxide solution. CSF samples (0.5 mL) were acidified with 50 µL of trifluoroacetic acid and extracted with chloroform. Alkaline precipitation of plasma proteins was preferred as under acidic conditions coelution of **1** with another substance was observed. The recoveries achieved were 60% for plasma and 78% for CSF. Separation was achieved with a normal phase analytical column, with 90% methanol : 10% 0.01 M sodium acetate (pH 4.6) as mobile phase. The LOD achieved with this method was 0.5 ng/mL for plasma and CSF samples (Unni *et al.*, 1989).

Miller and Verma developed a radioimmunoassay to study the time course of plasma concentrations and tissue distributions of **1** in rats (Miller and Verma, 1989). Lawrence and Yatim developed a method for the determination of **1** in plasma, whole blood and CSF samples (Lawrence and Yatim, 1990). Neostigmine was used as stabilizing agent. Sample pretreatment was achieved using solid-phase extraction (SPE) of samples adjusted to pH 4 with a 0.01 M citrate buffer on a cyano column. Elution was accomplished with 1.5 mL of 20% ACN in citrate buffer. Separation was achieved on a reversed phase analytical column with a mobile phase consisting of 8 % ACN : 92% 100 mM sodium citrate buffer (pH 4.0) with 0.02% sodium octylsulphate and 0.05% octylamine. The recovery of **1** was 99 ± 7% from standard solutions (pH 4) and 102 ± 4% from CSF. In the analysis of plasma samples, the use of methanol as precipitating agent gave better recoveries (82 ± 4%) than the use of perchloric acid (62 ± 4%). The LOD was 0.2 ng/mL, using ECD, which is four times higher than in the method reported by Whelpton and Moore (1985). On the other hand, the SPE procedure can be performed at acidic conditions, thus reducing the alkaline hydrolysis of **1** (Lawrence and Yatim, 1990).

Zhao *et al.* performed the determination of **1** and its major metabolite eseroline in rat plasma using a one-step SPE procedure and photodiode array (PDA) detection (Zhao *et al.*, 2003). This method was applied to study metabolic stability and pharmacokinetics of **1** in rats. Different SPE packing materials were compared. The best results were obtained using a Bond Elut C_{18} column with a recovery of 88 ± 3% at a concentration of 0.25 µg/mL. A reversed phase column was used for separation with 15 % ACN : 85 % 10 mM sodium dihydrogen phosphate

(pH 3.0) as mobile phase. The LOD of this method was 10 ng/mL for **1** and 25 ng/mL for eseroline using 0.1 mL of plasma (Zhao *et al.*, 2003).

2.2 Tacrine

Tacrine (**2**) was the first drug for the treatment of AD which was clinically tested in larger patient regimes. It was approved by the FDA in 1993 (Giacobini, 1998), but is now no longer in use because of severe side effects (Polinsky, 1998). **2** is a centrally acting reversible cholinesterase inhibitor. **2** was first synthesized by Albert and Gledhill in 1945 (Albert and Gledhill, 1945), but its pharmacology towards AChE was started being studied in 1986 by Summers *et al.* (Summers *et al.*, 1986). The structure of **2** is shown in Figure 2.2. Details for the most relevant methods developed for the analysis of **2** are given in Table 2.2.

2.2.1 Degradation studies

Sathyan *et al.* investigated the stability of **2** at room temperature and at 37 °C in both water and 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 over a period of 30 days. **2** was found to be very stable under both acidic and more basic conditions. In a study investigating the chemical degradation of **2** in poly(*n*-butylcyanoacrylate), Wilson *et al.* obtained comparable results (Wilson *et al.*, 2008). They investigated samples stored at room temperature (15–20 °C), refrigerated (3–5 °C), or at 37 °C (at a relative humidity of 75%) over a period of 3 months. **2** and the polymer were found to be physically and chemically stable and retained their pharmaceutical properties under these conditions over the tested period (Wilson *et al.*, 2008).

Marques *et al.* performed a stability study of **2** (Marques *et al.*, 2010a). No degradation products were observed after reflux in either 2 M hydrochloric acid (HCl) or 2 M sodium hydroxide (NaOH) solution for 5 h. However, when the drug was refluxed with 3% of hydrogen peroxide (H_2O_2) for 2 h, more than 50% of **2** were degraded. In this study, the bioactivity of the degradation products was also investigated. Part of the RPLC column eluate was split to an on-line continuous-flow AChE bioassay. Some of the degradation products formed under H_2O_2 treatment were found to inhibit AChE. The structures of tacrine (**2**) and the identified bioactive compounds are shown in Figure 2.2. The degradation products were identified by liquid chromatography–mass spectrometry (LC–MS) and NMR (Marques *et al.*, 2010a).

2.2.2 Metabolism studies

In vitro studies have shown that the cytochrome P450 enzyme 1A2 (CYP1A2) is the major enzyme involved in the oxidation of **2** (Hansen *et al.*, 1998). The main metabolic pathway involves hydroxylation of the saturated ring at the position 1, 2, and 4. These hydroxylated metabolites have been reported to be potent AChE inhibitors and to be active in various animal

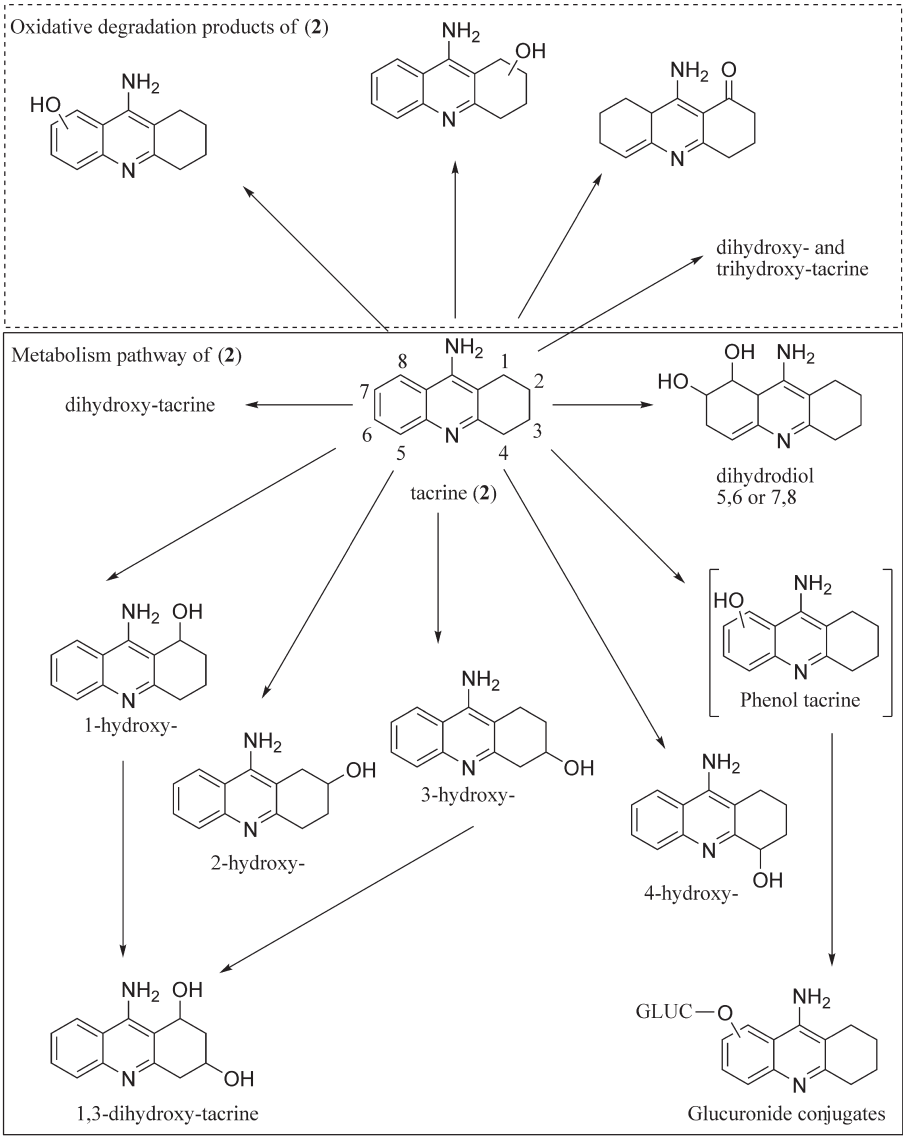


Figure 2.2 Metabolism and decomposition pathway of tacrine (**2**) (adapted with permission from Pool *et al.*, 1997 and Marques *et al.*, 2010a.

models (Hsu *et al.*, 1990a). Hsu *et al.* reported the urinary metabolic profiling of **2** in rat using UV detection for the quantification and direct-probe electron ionization MS and ^{1}H-NMR analysis for identification (Hsu *et al.*, 1990b). The sample cleanup involved LLE with 1:1 cyclohexane : ethyl acetate. Prior to identification, fractionation of the urine samples was performed on a semi-preparative analytical phenyl column. Each fraction was subsequently purified on a semi-preparative cyano column. This cleanup procedure resulted in clean samples without interferences for MS and ^{1}H-NMR analysis. In this way, the hydroxylated metabolites of **2** in urine were isolated and identified (Hsu *et al.*, 1990b).

Pool *et al.* used radio labeled [^{14}C]-tacrine to investigate the major excretion route of **2** in rats, dogs and humans (Pool *et al.*, 1997). Metabolic profiling and identification was achieved using gradient HPLC in combination with on-line radioactivity detection and thermospray (TSP) LC–MS. The sample cleanup procedure for urine samples was LLE with ethyl acetate; a typical recovery of 60% for all the metabolites was achieved. For plasma samples, a protein precipitation step with ethanol preceded the LLE (recovery ranged between 70-90%). Individual metabolites were isolated using a semi-preparative phenyl column with a linear gradient elution starting from 0% ACN : 50 mM aqueous ammonium formate (pH 3.1) to a final concentration of 20 % of ACN. Further purification was achieved with a cyano column. The purified fractions were analyzed by TSP-MS and, if sufficient sample was available, additionally by ^{1}H-NMR analysis. The major route of excretion was via the urine in all three species. Several mono-hydroxylated metabolites were found, involving hydroxylation at either the aliphatic or the aromatic ring. In addition, 1,3-, 5,6- and 7,8-dihydroxylated metabolites were observed, as well as glucuronic acid conjugates. The metabolic pathway of **2**, established in this study, is shown in Figure 2.2 (Pool *et al.*, 1997).

Bao *et al.* reported the use of a trapping-column system for the on-line trapping and preconcentration of the metabolites of **2** generated by dog liver microsomal incubations prior to analysis using MS and especially ^{1}H-NMR (Bao *et al.*, 2002). The metabolic mixture is separated by RPLC in the first column run. The metabolites are detected by a UV detector (320 nm for 1-hydroxy-tacrine). By valve switching, the analyte fraction is introduced onto the trapping column. After switching the valve again, the concentrated peak is back-flush eluted using a solvent containing 50% D_3-ACN into a NMR probe. This trapping-column system is especially useful if the amount of analyte is limited and when the chromatographic peaks are well separated (Bao *et al.*, 2002). By preconcentrating the peaks eluting from the HPLC, the data acquisition time of the NMR experiments can be reduced.

2.2.3 Bioanalytical studies

Ekman *et al.* developed a method to determine **2** and 1-OH-tacrine in human plasma (Ekman *et al.*, 1989). Sample preparation was carried out by extraction with dichloromethane. Recoveries for **2** and 1-OH-tacrine were higher than 93%. Separation was achieved with RPLC and UV detection. The LOD for this method was 0.3 ng/mL for both compounds. The method was used to determine **2** and 1-OH-tacrine in plasma of Alzheimer's patients (Ekman *et al.*, 1989).

Hsu *et al.* developed a RPLC method for the determination of **2** and three of its hydroxylated metabolites in rat plasma (Hsu *et al.*, 1990a). The cleanup was achieved with LLE using a 1:1 (v/v) mixture of cyclohexane : ethyl acetate. The recovery achieved was ~95% for tacrine, ~70% for 1-OH-tacrine, ~34% for 2-OH-tacrine, and ~84% for 4-OH-tacrine. The LOD was 1 ng/mL for all analytes using UV detection (Hsu *et al.*, 1990a). The mobile phase used was 70% ACN : 30% 50 mM ammonium acetate (pH 3.1).

In another study, Hadwiger *et al.* determined **2** and its metabolites in rat bile microdialysates (Hadwiger *et al.*, 1994). The in vivo microdialysis sampling is increasingly used in pharmacokinetic and metabolic profiling studies. **2** and its metabolites were isolated from the microdialysates using LLE with ethyl acetate achieving a recovery of >84% for **2** and of >93% for 1-OH-tacrine. The compounds were analyzed using RPLC with a 1 mm i.d. microbore column packed with a phenyl stationary phase and fluorescence detection (excitation and emission at 330 nm 365 nm, respectively). A LOD of 0.25 ng/mL was achieved for **2** in a 5 µL sample. Because of the low detection limits of the microbore LC–fluorescence method, **2** could be continuously monitored in the bile for more than 4 hours using microdialysis sampling (Hadwiger *et al.*, 1994).

Haughey *et al.* developed a method for the simultaneous determination of **2**, 1-OH-tacrine, 2-OH-tacrine and 4-OH-tacrine in human plasma (Haughey *et al.*, 1994). Plasma samples were alkalinized and extracted with a mixture of chloroform : 1-propanol (9 : 1, v/v). Recoveries ranged from 68-83% for **2** and its metabolites. The separation was achieved by RPLC on a cyano column. The mobile phase consisted of 30% ACN : 70% 10 mM sodium acetate buffer (pH 4.0), the column eluent was monitored using fluorescence detection (240 and 355 nm, excitation and emission). The LOD achieved with this method was 0.5 ng/mL for 2 and 4-OH-tacrine and 1 ng/mL for 2-OH-tacrine and 1-OH-tacrine. The sensitivity of the method was adequate for the determination of **2** and its metabolites after administration of 40 mg single dose of Cognex® (tacrine tablets) to healthy volunteers (Haughey *et al.*, 1994).

Aymard *et al.* developed a HPLC method with UV (239 nm) and fluorescence (330 and 360 nm excitation and emission wavelength) detection for the simultaneous determination of **2**, nimodipine and their respective metabolites in the plasma of AD patients (Aymard *et al.*, 1998). Sample preparation was carried out by using ACN as precipitating agent, followed by LLE with methyl tert.-butyl-ether : *n*-hexane (1:1). The recovery obtained was 67% for all compounds, except for 2-OH-tacrine for which it was 39%. Chromatography was carried out using a RP Shandon Hypersil phenyl column with 39% ACN : 61% 4.4 mM KH_2PO_4 buffer as mobile phase. The LOQ of the method was 0.3 ng/mL for **2** and its metabolites with 1 mL of plasma required (Aymard *et al.*, 1998).

Hansen *et al.* reported the determination of **2** and three metabolites in human plasma and urine (Hansen *et al.*, 1998). The method was based on LLE with ethyl acetate, isocratic RPLC, and fluorescence detection (330 and 365 nm for excitation and emission, respectively). The recoveries ranged from 84%-105% in plasma and from 64-100% in urine for all four compounds. The LODs achieved were 0.5 nM (0.11 ng/mL) for 2-OH-tacrine and 4-OHtacrine, and 2 nM (0.43 ng/mL) for 1-OH-tacrine and **2** in plasma, and 60 nM (12.9 ng/mL) for 2-OH-tacrine

and 4-OH-tacrine, 30 nM (6.4 ng/mL) for 1-OH-tacrine, and 80 nM (17.1ng/mL) for **2** in urine (Hansen *et al.*, 1998). In urine, the LODs were much higher than in plasma, which indicates that for urine a different cleanup method may be needed.

Chollet *et al.* developed a method for the therapeutic monitoring of **2** in human plasma (Chollet *et al.*, 2000). The method involved protein precipitation with ACN and a fast isocratic separation on a cyano column eluted in reversed-phase mode with 55% ACN : 45% 0.05 M NaH_2PO_4 (pH 7.0) as mobile phase. The entire sample preparation took place in an HPLC vial and no further liquid transfer was required. The mean overall recovery achieved was >87%. The LOD was in the order of 0.4 ng/mL, requiring 0.5 mL of plasma. The method was applied to the therapeutic monitoring of **2** over a 60 week treatment period in patients suffering from AD (Chollet *et al.*, 2000).

Jiang *et al.* developed a method for the simultaneous determination of *N*-butyramide-tacrine (BTHA) and **2** in mouse plasma and brain homogenate (Jiang *et al.*, 2003). BTHA was synthesized as a prodrug of 2. Sample preparation was done by protein precipitation with methanol. The recoveries for BTHA in plasma or brain at 250 ng/mL were 91% and 69%, respectively. The recoveries for **2** in plasma or brain at 25 ng/mL were 71% and 73%, respectively. Separation was carried out using RPLC, with UV detection (240 nm). The LOD of the method was 200 ng/mL for BTHA and 20 ng/mL for **2** in both matrices. The results of tissue distribution studies showed that the prodrug provided a promising approach for brain target delivery, with sustained brain levels of **2** and significantly reduced toxicity of the parent drug (Jiang *et al.*, 2003).

2.3 Donepezil

Donepezil (**3**) is an AChE inhibitor which was approved by the FDA in 1996 (Liang and Tang, 2004). It is a piperidine-based, reversible AChE inhibitor with a significantly lower affinity for butyrylcholinesterase (Scriabine, 2004). The drug is well tolerated with typical cholinergic side effects. Importantly, there has been no evidence of clinical changes in laboratory parameters, including liver function, as reported for **2** (Racchi *et al.*, 2004).

Donezepil (**3**) was first synthesized in 1992 by Sugimoto *et al.* (Sugimoto *et al.*, 1992). Soon, it was tested against AChE and found to be a highly selective inhibitor of the enzyme. Recently, a more economic process has been reported for a large-scale synthesis (Niphade *et al.*, 2008). Details for the most relevant methods developed for the analysis of **3** are given in Table 2.3.

2.3.1 Degradation studies

Pappa *et al.* validated a RPLC method to determine donepezil hydrochloride in tablets (Pappa *et al.*, 2002). The mobile phase was 50% methanol : 50% 0.02 M Na_2HPO_4 : 0.5% triethylamine (pH 2.7), and UV detection at 268 nm. The authors observed degradation of donepezil in 1 M HCl, 1 M NaOH and 30% of H_2O_2 under reflux. Degradation products were not characterized (Pappa *et al.*, 2002).

Yamreudeewong *et al.* described a method to determine the stability of **3** in an extemporaneously prepared oral liquid, that is a mixture of sorbitol and water, after 4 weeks storage at both room temperature (22–26 °C) and in the refrigerator (4–8 °C) (Yamreudeewong *et al.*, 2006). They applied a method described earlier (Pappa *et al.*, 2002). Donepezil (**3**) was found to be stable with changes in concentrations within ± 10% of the initial concentrations after a storage time of up to 4 weeks (Yamreudeewong *et al.*, 2006).

Kafkala *et al.* validated a RPLC method for determination of donepezil hydrochloride and its impurities in oral pharmaceutical formulations (Kafkala *et al.*, 2008). The separation was achieved using a mobile phase of 38% methanol : 62% 0.005 M K_2HPO_4 (pH 3.7). The elution was isocratic for the first 15 min and altered gradually to 62% methanol : 38% 0.005M K_2HPO_4 (pH 3.7) over 12 min. PDA detection was used for the spectrum and peak purity extraction while the analysis was carried out at 270 nm. A total of 4 impurities were separated. The structures of these impurities are shown in Figure 2.3 (Kafkala *et al.*, 2008).

2.3.2 Metabolism studies

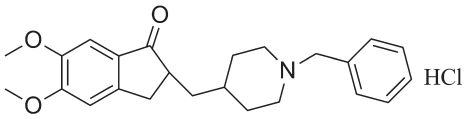
The metabolism of **3** takes place in the liver by the isoenzymes CYP2D6 and CYP3A4, which produce four major metabolites, corresponding to three metabolic pathways: *O*-demethylation to metabolites M1 and M2, with partial glucuronidation to metabolites M11 and M12, hydrolysis to metabolite M8 and *N*-oxidation to metabolite M5 and M6 (see Figure 2.4). Two of which are still active against AChE (M1 and M3) (Matsui *et al.*, 1999a, Patel *et al.*, 2008, Tiseo *et al.*, 1998).

Matsui *et al.* investigated the absorption, distribution, metabolism, and excretion (ADME) of ^{14}C labeled **3** in male Sprague-Dawley rats after a single oral administration (Matsui *et al.*, 1999a). Metabolic profiling was performed in plasma, brain, liver, and kidneys at 0.5 h after administration and in urine, feces, and bile, 24 h after administration. For identification purposes, the metabolites were first isolated by thin layer chromatography (*n*-butanol : acetic acid : water 4:1:1) and subsequently purified with RPLC (52% ACN : 48 % 5 mM SDS (pH 2.5). The structure elucidation was done using fast-atom bombardment MS and ^1H-NMR. The proposed metabolic pathway of **3** in rats is shown in Figure 2.4.

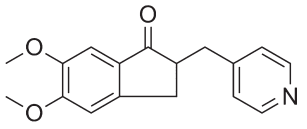
2.3.3 Bioanalytical studies

Haginaka and Seyama developed a method for determination of **3** enantiomers in rat plasma (Haginaka and Chikako, 1992). An ovomucoid (OVM)-bonded column could resolve both enantiomers of **3**. On the other hand, preliminary studies revealed that **3** and a plausible metabolite of **3** could not be resolved on the OVM-bonded column, so they developed a coupled achiral-chiral chromatographic method involving a trapping column. Detection was achieved with fluorescence detection at 318 nm and 390 nm as excitation and emission wavelength, respectively. The detection limits of **3** and the enantiomers were 1 ng/mL with an injection volume of 0.2 mL of deproteinized plasma samples (Haginaka and Chikako, 1992).

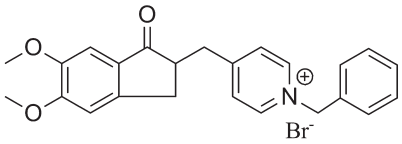
Matsui *et al.* developed a method to determine the enantiomers of **3** in human plasma (Matsui *et al.*, 1999b). The enantiomers show a slightly different extent of AChE inhibition.



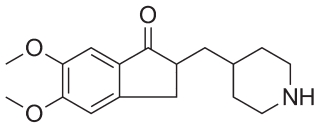
Donepezil Hydrochloride (**3**)



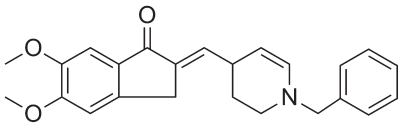
2-(4-pyridylmethyl)-5,6-dimethoxy-1-indanone



1-Benzyl-4-(5,6-dimethoxy-1-oxoindan) methyl pyridinium bromide



2-(4-Piperidinylmethyl)-5,6-dimethoxy-1-indanone **M4** of Figure 4



1-Benzyl-4-((5,6-dimethoxy-1-indanone)-2-yl) methylpiperidine-2-ene

Figure 2.3 Chemical structures of donepezil hydrochloride and its impurities (adapted with permission from Kafkala *et al.*, 2008).

Therefore, it is important to clarify the pharmacokinetics profiles of the individual isomers in a drug development program. To prevent the interconversion of donepezil enantiomers (via a keto-enol intermediate), a mild cleanup procedure was needed. LLE from 1 mL of plasma was achieved with 3% isopropanol in *n*-hexane (recovery ~90%). The enantiomers of **3** were separated and quantified using an avidin-conjugated column that is suitable for RPLC-MS analysis; the achieved LOQ for both enantiomers was 0.020 ng/mL. The mobile phase used was a solution of 25 % methanol : 75% 10 mM formic acid (Matsui *et al.*, 1999b).

Next to identifying the **3**- metabolites, as described in the metabolism section, Matsui *et al.* investigated the quantitative aspects of the ADME of **3** in male Sprague-Dawley rats after a single oral administration (Matsui *et al.*, 1999a). For this, quantitative determinations of **3** in plasma and brain were performed using RPLC and UV detection. With an assay validated within the ranges 1.0 to 200 ng/mL for plasma, and 2.0 to 500 ng/mL for brain homogenate samples, **3** and its metabolites were quantified in plasma, brain, liver, and kidneys 0.5 h after administration and in urine, feces, and bile for 24 h after administration (Matsui *et al.*, 1999a).

Xie *et al.* developed a method to determine **3** in human plasma using LC-MS/MS (Xie *et al.*, 2006). Sample preparation was carried out by alkalinizing the plasma with phosphate buffer (pH 14) followed by extraction with ethyl acetate. The recovery was higher than 65%. Chromatography was carried on a C_{18} reversed phase column. The substance was measured using

single reaction monitoring (SRM), employing the transition *m/z* 380 → 91. The lower limit of quantification (LLOQ) achieved for this method was 0.1 ng/mL. The method was applied to determine **3** in plasma during a pharmacokinetic study involving healthy volunteers orally receiving 5 mg of 3 (Xie *et al.*, 2006).

Radwan *et al.* developed a stereoselective HPLC method for the simultaneous determination of **3** enantiomers in tablets and plasma (Radwan *et al.*, 2006). Enantiomeric resolution was achieved on a Chiracel OD column using UV detection at 268 nm. The mobile phase consisted of 87% *n*-hexane : 12.9% isopropanol : 0.1% triethylamine. For sample preparation proteins were precipitated with ACN. Recoveries were higher than 93% for both enantiomers. The LOD achieved with this method was 20 ng/mL. The method was used to estimate the pharmacokinetic parameters of **3** enantiomers up to 12 h after oral administration in rats (Radwan *et al.*, 2006).

Apostolou *et al.* developed a method for the detection of **3** in human plasma using a completely automated 96-well format LLE system and subsequent LC-MS/MS detection (Apostolou *et al.*, 2007). The limit of quantification (LOQ) achieved with this method was 0.1 ng/mL.

In another study, Nakashima *et al.* increased the speed of the analysis of **3** in human and rat plasma as well as in blood and brain microdialysates by using a short C_{30} column in combination with fluorescence detection (Nakashima *et al.*, 2006). The mobile phase used was 27% ACN : 73% 25 mM citric acid/50 mM Na_2HPO_4 (pH 6.0) containing 3.5 mM sodium 1-octanesulfonate for plasma and 17% ACN : 3% methanol : 80% water containing 0.01% acetic acid was used as mobile phase for blood and brain microdialysates samples.

Yasui-Furukori *et al.* developed a simple method for the determination of **3** in human plasma, based on UV detection and the use of cisapride as internal standard (IS). Whereas the LOD and the selectivity of the method were rather poor (3 ng/mL), they were reported to be sufficient for the measurement of therapeutic concentration of **3** (Yasui-Furukori *et al.*, 2002).

Asakawa *et al.* reported a fully automated method for the detection of **3** in dog plasma (Asakawa *et al.*, 2007). Six HPLC pumps are used to deliver the solvents for washing, pretreatment and separation. Biological samples can be directly injected because an in-line filter performs the pretreatment of the samples. Furthermore, two C_{18} columns are positioned between the pumps and the auto sampler to purify the mobile phases. The typical LOD with this approach is 1 pg/mL of **3** in dog plasma (Asakawa *et al.*, 2007).

Patel *et al.* developed a method for the simultaneous analysis of **3** and its active metabolite 6-*O*-desmethyl-donepezil (6-*O*DD, metabolite M1 in Figure 2.4) in human plasma (Patel *et al.*, 2008). The sample pretreatment was done using SPE on Waters Oasis HLB cartridges. The mean recoveries obtained for **3** and 6-*O*DD were 62 % and 69 %, respectively. Donepezil (**3**) and 6-*O*DD in extracted plasma samples were stable for 96 h under refrigerated condition (−2 to 10 °C). The best RPLC in terms of resolution, speed, efficiency, and peak shape was achieved with a Novapak C_{18} column and a mobile phase consisting of 20 % methanol : 17% ACN : 63% 20 mM ammonium acetate (pH 3.43, 0.2% formic acid). MS in SRM mode was applied for the detection. An efficient RPLC separation is important because the two isomeric metabolites 5-*O*DD and 6-*O*DD have the same SRM transition (*m/z* 366.3 → 91.3). The LOQs achieved were

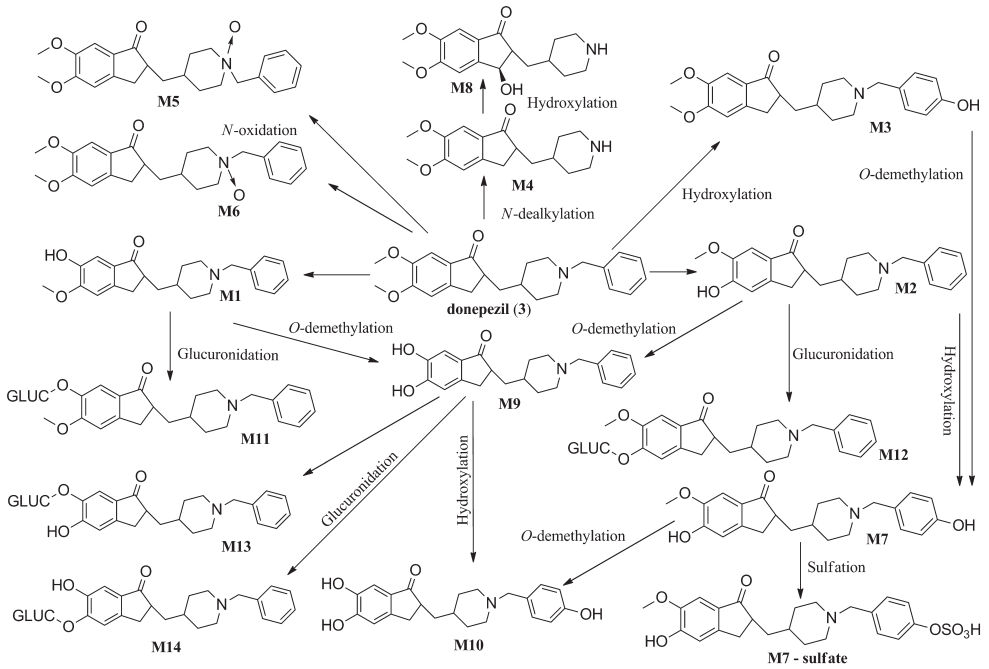


Figure 2.4 Proposed metabolic pathways of donepezil (**3**) (adapted with permission from Matsui *et al.*, 1999a).

0.10 ng/mL and 0.020 ng/mL for 3 and 6-ODD, respectively (Patel *et al.*, 2008). The same sample pretreatment method was later on applied by Shah *et al.* (Shah *et al.*, 2009). They achieved a 20% better recovery than Patel *et al.* by eliminating the evaporation and reconstitution step.

Yeh *et al.* applied field-amplified sampling stacking (FASS) in capillary electrophoresis (CE) for the quantitative analysis of **3** in human plasma (Yeh *et al.*, 2008). FASS is used to enhance sensitivity (Chien and Burgi, 1991). An amount of 200 µL of plasma was pretreated by LLE with 3% isopropanol : 97% *n*-hexane, providing a recovery better than 94% (measured at 3 concentration levels). The LOQ achieved with this method was 0.5 ng/mL (Yeh *et al.*, 2008).

2.4 Rivastigmine

Rivastigmine (**4**) is a pseudo-irreversible AChE inhibitor of the carbamate type. It was introduced for the treatment of mild to moderate dementia in the European Union in 1998 and in the US in 2000 (Bhatt *et al.*, 2007). It is synthesized using a very expensive chemical intermediate *N*-ethyl-*N*-methylcarbamoyl chloride in its synthesis (Mustazza *et al.*, 2002).

Mustazza *et al.* reported the synthesis of phenylcarbamates for good candidates of AChE in order to reduce the cost of the synthesis (Mustazza *et al.*, 2002). Details for the most relevant methods developed for the analysis of **4** are given in Table 2.4.

2.4.1 Degradation studies

Rao *et al.* developed a stability indicating LC method to determine the rivastigmine (**4**) hydrolysis product (S)-3-(1-dimethylaminoethyl) phenol (see Figure 2.5) in bulk samples (Rao *et al.*, 2005). The method involves the use of a RP-18 column and a mobile phase of 28% ACN : 72% sodium 1-heptane sulfonate (pH 3.0, adjusted with 0.01 M phosphoric acid).

Rivastigmine tartrate was found to be stable under all forced conditions tested (acidic, oxidative, elevated temperature and under irradiation), except in alkaline solution, where hydrolysis takes place (Figure 2.5). The method was fully validated to determine rivastigmine tartrate in bulk drug as well as in formulations (Rao *et al.*, 2005).

2.4.2 Metabolism studies

4 is rapidly and extensively metabolized via esterase-mediated hydrolysis of the carbamate moiety to the decarbamylated metabolite (S)-3-(1 dimethylaminoethyl)-phenol (NAP 226-90) (Figure 2.5) (Pommier and Frigola, 2003).

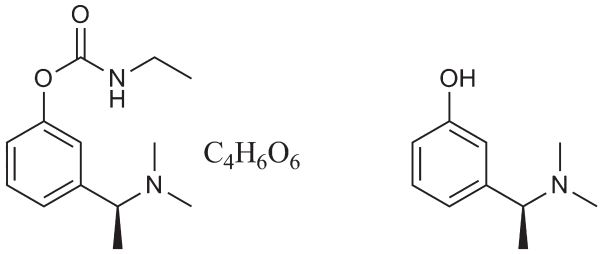
Lee *et al.* investigated the rate and the extent of absorption and metabolism of **4** after site-specific delivery of the drug in the gastrointestinal tract (GI) in humans using a naso-intestinal intubation technique (Lee *et al.*, 2004). The drug (3mg) was added to 10 mL water and the entire amount was administered through the tube to the specified site within the GI tract. It was observed that **4** was rapidly and equally well absorbed following specific delivery to upper and lower regions of the small intestine and from the ascending colon. The concentrations of NAP 226-90 were similar among different GI regions, suggesting that GI metabolism of **4** to its major metabolite NAP 226-90 was similar in different segments of the GI. However the authors suggested that a lower dose of **4** should be tested before a final conclusion can be made (Lee *et al.*, 2004).

2.4.3 Bioanalytical studies

Hossain developed a method to estimate the absolute bioavailability of **4** in patients with mild to moderate AD (Hossain, 2002). Plasma concentrations of **4** and its metabolite NAP 226-90 were measured with gas chromatography-mass spectrometry (GC-MS) (Hossain, 2002).

Pommier and Frigola developed a method to quantify **4** and the metabolite NAP 226-90 in human plasma (Pommier and Frigola, 2003). The cleanup procedure involved a LLE with methyl tert.-butyl-ether at basic pH and simultaneous derivatization of NAP 226-90 with propionic acid anhydride. The recovery was 115% for rivastigmine and 88% for its metabolite. The analysis was performed with RPLC coupled to atmospheric-pressure chemical ionization (APCI) MS. The LOQ of this method was 0.2 ng/mL for **4** requiring 0.5 mL of plasma (Pommier and Frigola, 2003).

Sha *et al.* developed a method for the quantification of **4** in canine plasma samples using headspace solid-phase microextraction (SPME) and capillary GC-MS with electron impact



Rivastigmine (**4**) (**NAP-226-90**)

Figure 2.5 Structure of rivastigmine tartrate (**4**) and its hydrolysis product NAP 226-90 (adapted with permission from Rao *et al.*, 2005).

ionization (EI) (Sha *et al.*, 2004). The aim was to develop a simple, fast and solvent-free extraction method for **4** from plasma. The LOQ of this method was also 0.2 ng/mL, also requiring 0.5 mL of plasma (Sha *et al.*, 2004).

Enz *et al.* developed a method for the simultaneous determination of **4** and its metabolite NAP 226-90 in rat plasma and brain homogenates (Enz *et al.*, 2004). **4** and NAP 226-90 were extracted from plasma and brain using ethyl acetate. Recoveries for **4** and NAP 226-90 were 93% and 95%, respectively, for both matrices. Separation was achieved by RPLC and ESI-MS detection. The LOQ achieved with this method was 0.13 ng/mL in plasma and 0.6 ng/g in brain for **4** and 0.17 ng/mL in plasma and 0.83 ng/g in brain for NAP 226-90. An amount of 0.1 mL of rat plasma was required (Enz *et al.*, 2004). The results of Enz *et al.* were comparable with the results obtained by the method of Pommier and Frigola (Pommier and Frigola, 2003), while both applied mass spectrometric detection with different ionization technique ESI and APCI.

Bhatt *et al.* developed a method for the detection of **4** in human plasma which they claim to reduce sample preparation and analysis time (Bhatt *et al.*, 2007), what remains questionable as the method involved SPE as sample pretreatment. The recovery achieved was 86.3%. The separation was carried out using RPLC and the total run time for each sample analysis was 2.0 min which is a 4-times reduction compared to Sha *et al.* (Sha *et al.*, 2004). With positive-ion electrospray (ESI) MS detection, the LOQ of this method also was 0.2 ng/mL (Bhatt *et al.*, 2007).

Frankfort *et al.* reported a method for the determination of **4** and its metabolite NAP 226-90 in human plasma. The aim of the work was to further simplify the cleanup procedure. Using protein precipitation with methanol only, recoveries of 70.9% for **4** and 85.7% for NAP 226- 90 were achieved (Frankfort *et al.*, 2006).

Karthik *et al.* developed a method for determination of **4** in rat plasma (Karthik *et al.*, 2008). The chromatographic separation was achieved by RPLC, using a monomeric C_{18} column with a mobile phase consisting of 35% ACN : 75% 20 mM phosphate buffer (pH 3.0). The compound was detected using fluorescence detection at 220 nm and 293 nm as excitation and emission wavelengths, respectively. The LOD of this method was 25 ng/mL. The method was applied in

a pharmacokinetic study of **4** in rats (Karthik *et al.*, 2008).

Hsieh *et al.* developed a method for the simultaneous determination of **4**, NAP 226-90 and **5** in human plasma (Hsieh *et al.*, 2009). The method is based on the use of micellar electrokinetic chromatography (MEKC), first introduced by Terabe (Terabe, 2008). The sample cleanup using LLE with diethylether provided recoveries of 90 and 99% for **4** at 2 and 50 ng/mL, respectively, and of 90 and 100.8% for NAP 226-90 at 2 and 50 ng/mL, respectively. UV detection was applied at 214 or 200 nm. A 20% ACN : 25 mM Tris buffer (pH 5.0) with 160 mM sodium octansulfonate (SOS) : 0.01% poly(vinylpyrrolidinone) was chosen as the separation buffer in MEKC (Hsieh *et al.*, 2009).

2.5 Galantamine

Galantamine (**5**) is the most recently approved drug for the treatment of AD by the FDA in 2001 (Hoffmann and Hock, 2004). It is a tertiary alkaloid, isolated from the bulbs and flowers of Caucasian snowdrop (*Galanthus woronowii*, Amaryllidaceae) (see Figure 2.6). The cost of obtaining galantamine from natural sources (Narcissus species) (de Jong *et al.*, 2006) is very high, and a total synthesis procedure via (2)-narwedine has been extensively worked out for large-scale industrial production (Chang *et al.*, 2010, Satcharoen *et al.*, 2007).

Galantamine (**5**) started in the market with the commercial name Reminyl which was changed in 2005 to Razadyne) (Berkov *et al.*, 2008, Leonard *et al.*, 2005). Details for the most relevant methods developed for the analysis of **5** are given in Table 2.5.

2.5.1 Degradation studies

The stability of **5** in methanol spiked with 2% bovine serum albumine (BSA), in human plasma and whole blood was studied by Verhaeghe *et al.* (Verhaeghe *et al.*, 2003). In human plasma, **5** was found to be stable for 72 h at room temperature and in human blood for 72 h at 40 °C. In methanol spiked with 2% BSA, stability of **5** was shown for 6 months at -20 °C, for 1 month at 4 °C, and for 3 days at room temperature (Verhaeghe *et al.*, 2003).

Leonard *et al.* conducted an accelerated stability study for two salts of **5**: the lactate and the hydrobromide, the latter being the one that is used in the commercial drug. The compound degradation was monitored at three different pH values (2.5, 5.2, and 10.0), at three different temperatures (25, 40, and 80 °C) after 1, 7, and 28 days as well as after 2 months. It was found that the lactate exhibits a comparable or superior stability when compared to the hydrobromide under the conditions tested. Degradation was observed after two months of storage at high temperature and in combination of extreme pH (2.5 and 10.0). For instance, both hydrobromide and lactate showed a decrease in purity to 31.6% and 29.5%, respectively, after 2 months at pH 10 and 80 °C (Leonard *et al.*, 2005).

Visky *et al.* applied CE-ESI-MS/MS to evaluate the impurity profile of the hydrobromide

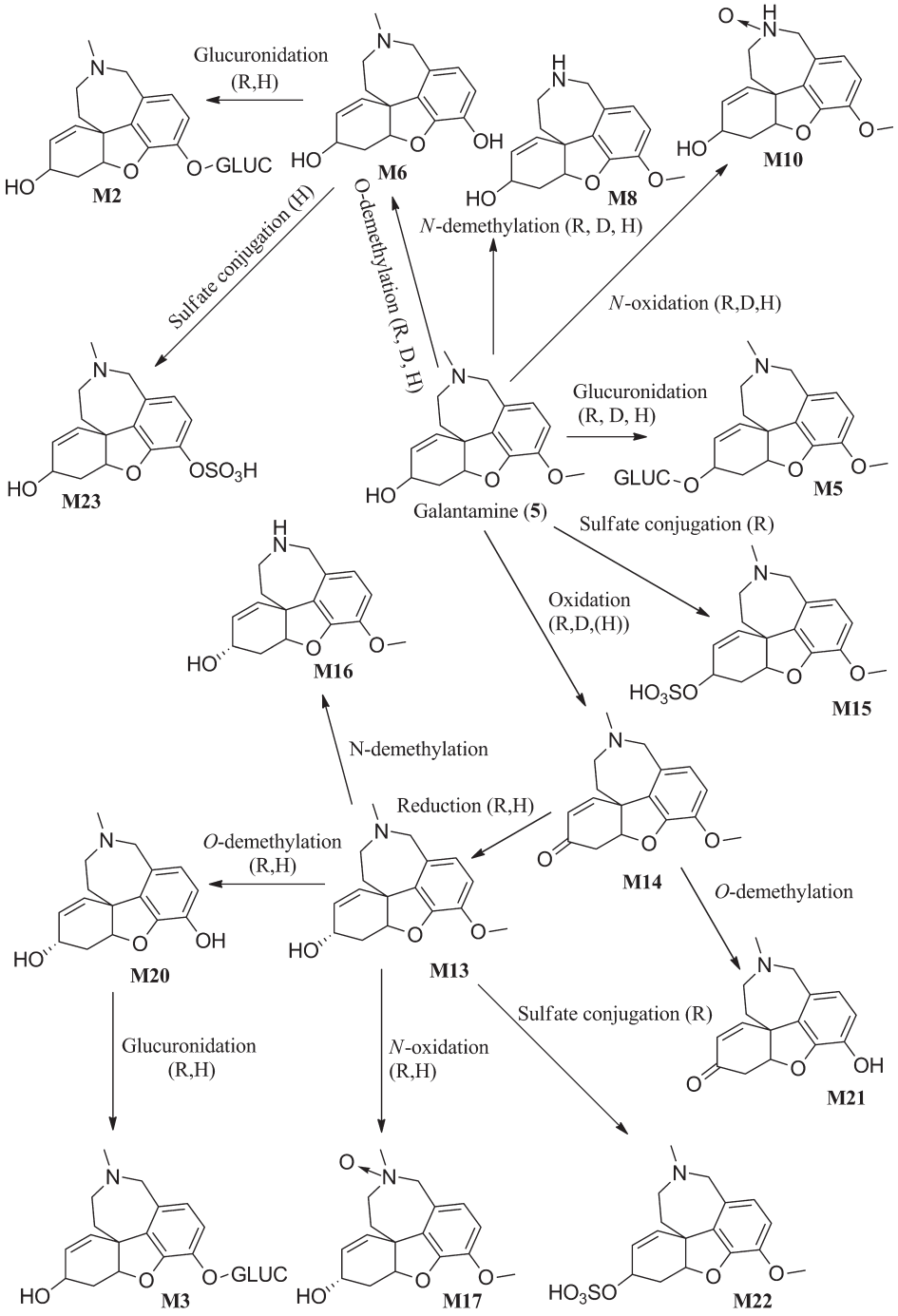


Figure 2.6 Metabolic pathways of galantamine (**5**) after a single oral dose, GLUC glucuronic acid, R, H and D, refer to the metabolites found in rat, human and dog (adapted with permission from Mannens *et al.*, 2002).

in stressed Reminyl extended release capsules (Visky *et al.*, 2005). Reminyl capsules were stored in stability chambers under different conditions: 25 °C / 60% relative humidity (RH) for 18 months; 30 °C / 70% RH for 18 months; 40 °C for 6 months moreover the capsules were irradiated for 8 h. The sample preparation was done by weighting an accurate amount of the drug, and dissolving it with a solution of methanol : water (40 : 60, v/v), and ultrasonicating it for 5 min. Afterwards, the mixture was shaken mechanically for 30 min and the solution was filtered through a 0.45 μm filter. The authors compared the method with RPLC and also with CE-chiral-UV. The RPLC analysis was performed using a C_{18} analytical column, employing the following eluents: A was 95% 30 mM phosphate buffer pH 6.5 : 5% methanol, eluent B was 95% ACN : 5% methanol. Detection was accomplished using UV at 230 nm. The CE chiral- UV was performed in an uncoated fused-silica capillary. The background electrolyte composition was 20 mM α-cyclodextrin dissolved in 50 mM phosphate buffer, pH 3.0. The UV detection was performed at 214 nm. The CE-ESI-MS/MS analysis was also performed in uncoated fused-silica. The background electrolyte composition was 25% ACN : 25% methanol : 50% 100 mM ammonium acetate. The detection was performed in positive ESI mode. Both by CE and RPLC, two impurities were detected in the stressed samples (18 months, 25 °C / 60% RH) at concentrations lower than 0.05%. The identification of these impurities was done using CE-MS/MS. The unknown compounds involve a methylation at the azepine nitrogen corresponding to the formation of a quaternary ammonium derivative of **5**, the second substance corresponds to *O*-desmethyl galantamine (structure M6 in Figure 2.6) (Visky *et al.*, 2005).

2.5.2. Metabolism studies

5 is extensively metabolized in the liver by CYP2D6 and CYP3A4, which are the same isoenzymes involved in the metabolism of **3** (Malakova *et al.*, 2007, Mannens *et al.*, 2002). The principal metabolic pathways are *O*-demethylation, *N*-demethylation, *N*-oxidation, epimerization, glucuronidation, and sulfate conjugation. The metabolic pathway of galantamine is shown in Figure 2.6 (Mannens *et al.*, 2002). The metabolites are either pharmacologically inactive or less when compared to **5**.

2.5.3. Bioanalytical studies

Claessens *et al.* developed a method for the determination of **5** in human serum, in which normal-phase LC with UV detection at 235 nm was applied. The sample cleanup was achieved by LLE with dichloromethane (DCM), resulting in a recovery of 100.2%. The LOD of **5** in serum was 5 ng/mL, which was adequate for the study carried out (Claessens *et al.*, 1983)

Tencheva *et al.* reported the determination of **5** and its metabolites epigalantamine (M13 in Figure 2.6) and galantaminone (M14) in human plasma and urine (Tencheva *et al.*, 1987). Using LLE with chloroform, a recovery of 94-100% was obtained. In this work, RPLC with UV detection at 280 nm was applied. The LOD in urine and plasma was 50 ng/mL, which is ten times higher than the LOD found in serum by Claessens *et al.* However, the precision (RSD) of the method of Tencheva *et al.* was with 5% much better than the 37.8% achieved by Claes-

sens *et al.* The method was applied in a pharmacokinetic study. Blood samples were taken 180 min after the oral administration of 10 mg of Nivalin (old brand name used for commercial **5**) tablets. Concentrations of 3.2 μg/mL for **5** and 0.74 μg/mL for epigalantamine were detected in plasma. Urine samples were taken 12 h after subcutaneous administration of the drug. In urine, 0.98 μg/mL of **5**, 2.54 μg/mL of epigalantamine, and 0.66 μg/mL of galantaminone were detected (Tencheva *et al.*, 1987).

Verhaeghe *et al.* described a method for the analysis of **5** in heparinised human plasma (Verhaeghe *et al.*, 2003). RPLC with positive-ion ESI-MS was used. Initially, the sample cleanup was done using a mixed-mode SPE, but under these conditions the *N*-oxide metabolite (M10 in Figure 2.6) is co-extracted and partially converted back to **5** during the evaporation step.

Therefore, LLE with toluene at pH 13 was used, resulting in a recovery of **5** better than 90%. The LOQ was 1 ng/mL for 5. The method was applied to the analysis of 3000 clinical samples. Despite the manual cleanup step, 750 samples could be analyzed by two lab technicians in 4 working days. The isotopically labeled IS enabled short chromatographic run times in LC-MS (2.0 min) (Verhaeghe *et al.*, 2003).

Malakova *et al.* developed a method for the simultaneous determination of **5** and its phase-I metabolites in rat plasma, liver and brain (Malakova *et al.*, 2007). The method was validated using RPLC and fluorescence detection (excitation 280 nm, emission 310 nm). The identity of **5** and its metabolites in the biological samples was confirmed using RPLC-PDA-MS analysis. In this case, a sample cleanup procedure based on a mixed-mode cation-exchange reversed phase polymeric sorbent was applied. The phenomenon of the conversion of *N*-oxide galantamine back to **5** as described by Verhaeghe *et al.* was not observed in this study, as verified by the analysis of samples after the addition of galantamine *N*-oxide to drug free plasma. The recovery achieved was 81% for **5**, 57.8% for *O*-desmethyl galantamine, 49.7% for *N*-desmethyl-galantamine and 81.3% for epigalantamine. The LOD was 0.03 μM (8.6 ng/mL) for **5** in rat plasma samples. The method was applied in pharmacokinetic studies of **5** in rat plasma, liver and brain. Typical chromatograms for extracts of a spiked blank rat liver tissue and of a rat liver tissue 30 min after administration of 10 mg/kg galantamine hydrobromide are shown in Figure 2.7 (Malakova *et al.*, 2007).

Nirogi *et al.* developed a method for quantification of **5** in human plasma by RPLC MS in the SRM mode using the transitions *m/z* 288 → 213 for **5** and *m/z* 383 → 337 for loratadine (IS). The LOQ was 0.5 ng/mL (Nirogi *et al.*, 2007).

Hsieh *et al.* developed a method for the simultaneous determination of **4**, NAP 226-90 and **5** in human plasma (Hsieh *et al.*, 2009). The sample cleanup using LLE with diethylether provided recoveries of >85% for 5. UV detection was carried out at 214 or 200 nm. A mobile phase consisting of 20% ACN : 25 mM Tris buffer (pH 5.0) with 160 mM sodium octansulfonate (SOS) : 0.01% poly(vinylpyrrolidinone) was chosen as the separation buffer in MEKC. The LOD achieved with this method for **5** was 0.25 ng/mL. The method was applied for monitoring **5** or **4** and the metabolite NAP 226-90 in plasma samples of 11 AD patients after oral administration of the commercial products Reminyl (8 mg **5**) or Exelon (3 mg **4**).

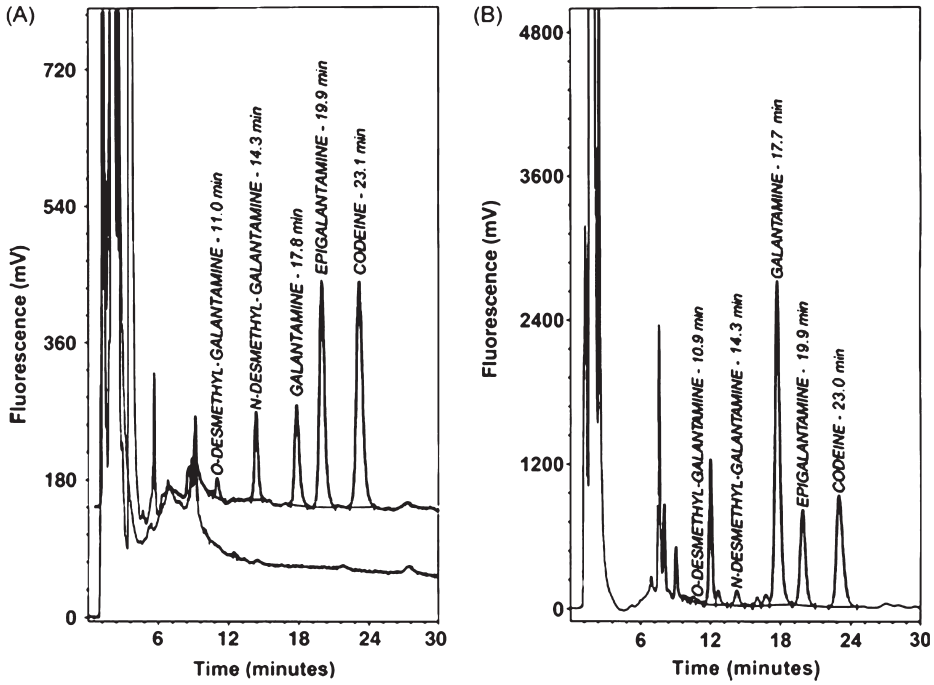


Figure 2.7 Chromatograms of galantamine (**5**) and its metabolites in rat liver tissue extracts: (A) a blank sample (lower chromatogram) and the drug-free rat liver tissue homogenate spiked with **5**, epigalantamine, *O*-desmethyl- galantamine, *N*-desmethyl-galantamine, and codeine (IS). (B) Chromatogram of the extract from the rat liver tissue 30 min after a single intramuscular dose of 10 mg/kg of galantamine hydrobromide (adapted with permission from Malakova *et al.*, 2007).

2.6 Huperzine A

Huperzine A (**6**), an alkaloid isolated from the Chinese herb Huperzia serrata, is a potent and reversible AChE inhibitor which crosses the blood-brain barrier smoothly and shows high specificity. The structure of **6** is shown in Figure 2.8. Compared with **2**, **3**, **4** and **5**, **6** has better penetration through the blood-brain barrier, higher oral bioavailability and longer duration of AChE inhibitory action (Wang *et al.*, 2006). It has been approved as a drug for AD treatment in China, and is marketed in the US as a dietary complement (Ye *et al.*, 2005). Various studies have been reported on the isolation and quantification of huperzine A in plant extract (Ma and Gang, 2008, Toribio *et al.*, 2007, Wu and Gu, 2006). The first total synthesis of **6** was reported by Qian and Ji in 1989 (Qian and Ji, 1989). **6** can also be synthesized in several other ways (Haudrechy *et al.*, 2000, Lee *et al.*, 2002). Details for the most relevant methods developed for the analysis of **6** are given in Table 2.6.

2.6.1 Degradation studies

Li *et al.* investigated the stability of **6** at **4** different conditions: in untreated plasma at room temperature for 4 h, in treated plasma in an autosampler vial at 4 °C for 10 h, at -20 °C for 2 weeks, and after three freeze-thaw cycles at -20 °C (Li *et al.*, 2008). **6** was found to be stable under all these conditions. In this study, quantification of **6** was carried out by positive ESI LC-MS/MS, with pseudoephedrine hydrochloride as IS. LLE with ethyl acetate yielded a recovery of 83 % for 0.1 ng/mL of **6** in plasma. Separation was achieved using a C_{18} column and 85% methanol : 15% water (0.2% formic acid) as the mobile phase. The LOQ was 0.05 ng/mL using 1 mL of plasma (Li *et al.*, 2008).

Similar studies of **6** in plasma and CSF for 60 days at -20 °C, involving freeze / thaw cycles, and for 24 h in the autosampler at room temperature, reported by Pan *et al.*, showed good stability of **6** under all investigated conditions for both biological matrices (Pan *et al.*, 2009).

Ashani *et al.* showed that under the following stress conditions: pH, temperature, ionic strength, different solvents **6** was basically stable (Ashani *et al.*, 1992).

Marques *et al.* studied the stability of **6** under irradiation, **6** was significantly transformed to a new compound called photohuperzine A (Marques *et al.*, 2010b). Its activity against AChE was found to be 100 times lower when compared to **6**. The structures of **6** and its photodegradation product are shown in Figure 2.8.

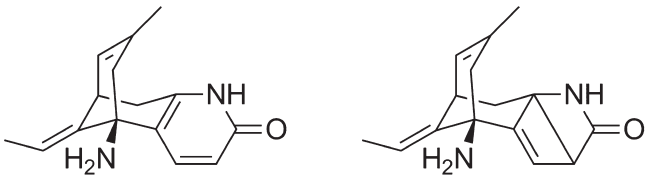
2.6.2 Metabolism studies

The metabolism of **6** in rat liver microsomes is mediated primarily by CYP1A2, with a probable secondary contribution of CYP3A12. CYP2C11 and 2E1 are likely not involved in the metabolism of **6** (Xiaochao *et al.*, 2003). Garcia *et al.* identified and characterized the major metabolite of huperzine A in rat blood (Garcia *et al.*, 2004). The compound was isolated from blood and liver samples and subsequently analyzed by ESI-MS and ^{1}H-NMR spectroscopy. The metabolite was identified to be 13,14-epoxy huperzine (Garcia *et al.*, 2004).

2.6.3 Bioanalytical studies

Wang *et al.* reported the determination of **6** in dog plasma, using huperzine B as IS (Wang *et al.*, 2004). The analysis was performed using a C_{18} column a mobile phase of 35% ACN : 40% methanol : 25% 10mM ammonium acetate, and positive-ion ESI-MS detection. The total run time was 2 min allowing a high sample throughput in pharmacokinetics studies. An LOD as low as 0.01 ng/mL was achieved, enabling the analysis of dog plasma samples from sustained-release formulations of **6** (Wang *et al.*, 2004).

Wei *et al.* developed a method for the simultaneous determination of **6** and ZT1 a prodrug of **6** in plasma (Wei *et al.*, 2006). The separation was achieved by RPLC with UV detection (313 nm). The rat plasma was extracted with a solution of chloroform : isopropyl alcohol (9 : 1, v/v). The recovery achieved for ZT1 and **6** at 0.5 nmol/mL was 92% and 102%, respectively. The LOQ achieved with this method was 0.02 nmol/mL (20 nM) for both compounds (Wei *et al.*, 2006).



huperzine A (**6**) photohuperzine A

Figure 2.8 Structure of huperzine A (**6**) and its photodegradation product photohuperzine A (adapted with permission from Marques *et al.*, 2010b).

Earlier Li *et al.* developed a LC-MS/MS based method for the two compounds (Li *et al.*, 2004). Due to the rapid degradation of ZT1 a $NaBH_4$ based hydrogenation of ZT1 to its stable form rZT1 was applied, following blood sampling. The compounds were extracted by LLE using ethylacetate : isopropanol (950 : 50, v/v). The separation was achieved using a mobile phase consisting of methanol : water, containing 0.05% ammonium formate. The ion trap MS was operated in MS/MS full scan mode, while specific transitions were applied. The achieved LLOQ was 0.12 ng/mL (0.29 nM) for ZT1 and 0.51 ng/mL (2.1 nM) for **6**. The method was applied to a pharmacokinetics study in rats (Li *et al.*, 2004).

Li *et al.* developed a method for the determination of **6** in human plasma also using LC-MS (Li *et al.*, 2007). Codeine phosphate was used as IS, which is more readily available than the synthetic huperzine B, used by Wang *et al.* The run time was 2 min and the LOD 0.064 ng/mL. The method was applied in pharmacokinetics studies (Li *et al.*, 2007).

Hameda *et al.* described the use of matrix-assisted laser desorption ionization time-of-flight MS (MALDI-TOF-MS) and CE with laser induced fluorescence detection (CE-LIF) for the determination of rhodamine-huperzine A complex in human serum (Hameda *et al.*, 2007). The compound was labeled with rhodamine B isothiocyanate (RBITC) resulting in a highly fluorescent complex. The isothiocyanate group of RBITC reacts with amino groups. The MALDI-TOF-MS technique was applied for the quantitative determination of the complex in serum using DHB (2,5-dihydroxybenzoic acid) as matrix. The LOD achieved was 20 nM. MALDI-TOF-MS is highly suitable for high-throughput trial sampling, but for more precise quantitative bioanalysis a more reproducible technique should be used. This explains the use of CE-LIF for the quantification of the complex in human serum. The LOD with CE-LIF was 4 nM. With MALDI-TOF, the detection limit was 5 times lower.

Yue *et al.* reported the determination of **6** in rat plasma (Yue *et al.*, 2007a) and cerebrospinal fluid (CSF) (Yue *et al.*, 2007b), based on LC with fluorescence detection. The rat plasma samples (200 µL) were extracted with chloroform after addition of an IS and 100 µL of a borax-sodium carbonate buffer (pH 11.8). A C_8 column was used, with a mobile phase of 45 % methanol : 55% water : 0.05% triethanol amine. CSF samples were analyzed without pretreatment. In this case, the separation was achieved with a C_{18} column employing a mobile phase of 60% methanol : 40% water : 0.05% triethanolamine. Huperzine A (**6**) was detected using a

fluorescence detector (310 nm excitation, 370 nm emission) for both matrices.

An LOQ of 6.25 ng/mL was achieved for rat plasma, following intranasal administration (Yue *et al.*, 2007a). Subsequently, the levels of **6** were investigated in both rat plasma and CSF after three ways of administration (intravenous, intranasal and intragastric) in order to decide which route is more efficient in transferring the drug into the CNS. It was observed that after intranasal administration, the maximum concentration cmax in the CSF is not reached as fast and as high as for the intravenous application route. However, 30 min after drug administration (167 μg/kg), the intranasal route showed slightly higher concentrations in the CSF than the intravenous route (Yue *et al.*, 2007b)

2.7 Conclusions

This review gives an overview of analytical methods applied for the detection of AChE inhibitors and their metabolites in different matrices. Most of these methods were developed for pharmacokinetic applications, or stability indicating studies. Especially in the first case speed, reliability, and applicability are the most important criteria. These parameters were obtained using mainly RPLC coupled with MS, especially in the past ten years when the coupling of these two techniques became more and more popular. Fluorescence detection also provided a robust and highly sensitive detection principle, what was basically shown for all of the described substances in various matrices. In terms of sample cleanup procedures applied for the discussed analytes, mainly LLE was used, which provided good recoveries. Another popular methodology for sample pretreatment purposes was SPE on various different materials. Future developments will certainly imply the application of modern technologies in HPLC, such as ultra-performance liquid chromatography, which can provide fast separations with high resolving power, also being ideal for large numbers of samples, particularly in pharmacokinetics studies.

Table 2.1 Summary of the most relevant methods developed for the determination of physostigmine (**1**) and its metabolites in different biological matrices. a if not stated otherwise the given values refer to all listed substances and conditions. Abbreviations are listed in the abbreviation list.

Substance	Matrix	Sample preparation	Separation
Physostigmine (**1**)	Human plasma, urine, whole blood	Plasma and blood: LLE (diethyl ether) Urine: LLE (benzene)	Spherisorb (150 × 4.6 mm, 3 µm) pH partition studies: 90% methanol - 10% ammonium nitrate buffer pH 8.6 biological extracts: 45% methanol - 45% ACN - 10% NH_4NO_3 pH 8.9.
1	Human plasma	LLE (methyl-tert.-butyl ether)	Spherisorb 5 ODS1 (125 × 4.9 mm, 5 µm) 95% ACN-5% 0.01 M sodium acetate
1	Human plasma	LLE (dichloro-methane)	Altex ultrasphere-Si (250 × 4.6 mm, 5 µm) 20% ACN - 80% 0.01 M NaH_2PO_4 and 2.5 mM tetramethylammonium (pH 3.0)
1	Human plasma, CSF	Plasma: LLE (diethyl ether) CSF: LLE (chloroform)	Spherisorb silica column (150 × 4.6 mm, 5 µm) 90% Methanol - 10% 0.01 M sodium acetate (pH 4.6)
1, Eseroline	Plasma, whole blood, CSF	SPE (cyano column)	Spherical C_{18} (100 × 4.6 mm, 3 µm) 8% ACN - 92% 0.10 M sodium citrate buffer pH 4.0 with 0.02% sodium octylsulphate and 0.05% octylamine.
1, Eseroline	Rat plasma	SPE (C_{18} column)	Alltech Ultrasphere silica (250 × 4.6 mm, 5 µm) 15% ACN - 85% 0.01 M NaH_2PO_4 (pH 3.0)

	Detection	Calibration range[a]	LOD/LOQ[a]	Recovery[a]	Reference
	Electrochemical	0.1–10 ng/mL	LOD Plasma: 50 pg/mL Whole blood: 100 pg/mL Urine: 25-50 pg/mL	nd	Whelpton *et al.*, 1985
	Fluorescence (254 and 346 nm excitation and emission)	0.1–3.0 ng/mL	LOD: 0.1 ng/mL	90.4 – 94.2%	Brodie *et al.*, 1987
	Fluorescence (240 and 360 nm excitation and emission)	0.5–25 ng/mL	LOD: 0.1 ng/mL	91.5 – 93.1%	Elsayed *et al.*, 1989
	Electrochemical	Plasma: 0.5–20 ng/mL CSF: 0.5–40 ng/mL	LOD Plasma: 0.5 ng/mL CSF: 0.5 ng/mL	Plasma: 60% CSF: 78%	Unni *et al.*, 1989
	Electrochemical	0.5–10 ng/mL	LOD: 0.2 ng/mL	Plasma: **1**: 82% CSF: **1**: 102% Blood: **1**: 20%	Lawrence and Yatim, 1990
	UV (246 nm)	0.1–2.5 μg/mL	LOD **1**: 10 ng/mL Eseroline: 25 ng/mL LOQ: **1**: 75 ng/mL Eseroline:100 ng/mL	**1**: 88% Eseroline: 61%	Zhao *et al.*, 2003

Table 2.2 Summary of the most relevant methods developed for the determination of tacrine (**2**) and its metabolites in different biological matrices. a if not stated otherwise the given values refer to all listed substances and conditions. Abbreviations are listed in the abbreviation list.

Substance	Matrix	Sample preparation	Separation
Tacrine (**2**), 1-OH-tacrine	Human Plasma	LLE (dichloro-methane)	Nucleosil C_{18} (150 × 4.6 mm, 5 µm) 20% ACN : 80% 20 mM phosphate buffer (pH 2.7)
2, 1-OH-tacrine, 2-OH-tacrine,4-OH-tacrine	Rat plasma	LLE (cyclohexane -ethyl acetate)	Shandon hypersil phenyl (100 × 4.6 mm, 3 µm) 70% ACN : 30% 0.02 M ammonium formate (pH 2.8)
2, 1-OH-tacrine, 2-OH-tacrine,4-OH-tacrine	Rat urine	LLE (cyclohexane -ethyl acetate)	Shandon hypersil phenyl (100 × 4.6 mm, 3 µm) 70% ACN : 30% 0.05 M ammonium formate (pH 3.1) Purification (2 columns): Shandon hypersil semi-prep phenyl (250 × 7.0 mm, 5 µm) (same mobile phase as above) Shandon CPS hypersil semi-prep cyano (250 × 7 mm, 5 µm) 70% ACN : 30% 0.25 M sodium acetate (pH 4)
2, 1-OH-tacrine	Rat bile micro dia-lysates	LLE (ethyl acetate)	SepStiks (100 × 1 mm, 5 µm for phenyl and 8 µm for cyano) 10 % methanol-5% ACN : 85 % 0.05 M ammonium phosphate (pH 2.5)
2, 1-OH-tacrine, 2-OH-tacrine, 4-OH-tacrine	Human plasma	LLE (chloroform-1-propanol)	Brownlee-Spheri-5 cyano (100 × 4.6 mm, 5 µm) 30% ACN : 70% 10 mM sodium acetate (pH 4.0)

	Detection	Calibration range[a]	LOD/LOQ[a]	Recovery[a]	Reference
	UV (240 nm)	nd	LOD: 0.3 ng/mL	93%	Ekman *et al.*, 1989
	UV (240 nm)	1-500 ng/mL	LOD: 1 ng/mL	2: 96% 1-OH THA: 70% 2-OH-THA: 35% 4-OH-THA: 84% (50 ng/mL)	Hsu *et al.*, 1990a
	UV (325 nm), electron impact EI/MS, ^{1}H-NMR	nd	nd	nd	Hsu *et al.*, 1990b
	Fluorescence (330 and 365 nm excitation and emission)	1-200 ng/mL	LOD 2: 0.25 ng/mL	2: >84% 1-OH tacrine: >93%	Hadwiger *et al.*, 1994
	Fluorescence (240 and 355 nm, excitation and emission)	2 and 4-OH-tacrine: 0.5 - 30 ng/mL 2-OH-tacrine: 1.0 - 30.0 ng/mL 1-OH-tacrine: 0.93 - 46.2 ng/mL	LOQ 2 and 4-OH-tacrine: 0.5 ng/mL 2-OH-tacrine: 1.0 ng/mL 1-OH-tacrine: 0.9 ng/mL	68% - 83%	Haughey *et al.*, 1994

Table 2.2 cont.

Substance	Matrix	Sample preparation	Separation
2, 1-OH-tacrine, 2-OH-tacrine, 4-OH-tacrine, 1,3-dihydroxy-tacrine, dihydrodiol, 7-OH-tacrine	Urine, feces and plasma from dog, rat and human	Plasma: precipitation with ice cold ethanol Urine: LLE (ethyl acetate)	Hypersil phenyl (250 × 6.2 mm, 5 µm), Gradient, 0% ACN : 100% 50 mM aqueous ammonium formate (pH 3.1)increasing to 20 % ACN in 60min, held for 30 min
2, 1-OH-tacrine, 2-OH-tacrine, 4-OH-tacrine	Human plasma	Precipitation with ACN, followed by LLE (methyl-tert.-butyl ether : *n*-hexane)	Shandon Hypersil phenyl (250 × 4.6 mm, 5 µm) 39 % ACN : 61 % 4 mM KH_2PO_4 buffer
2, 1-OH-tacrine, 2-OH-tacrine, 4-OH-tacrine	Human plasma, urine	LLE (ethyl acetate)	LiChrospher 60 RP select- B (250 × 4 mm, 5 µm) 13 % ACN : 87 % 0.2 M acetate buffer (pH 4)
2	Human plasma	Protein precipitation with ACN	Nucleosil cyano (125 × 4.0 mm, 5 µm) 55% ACN : 45% 0.05M NaH_2PO_4 buffer (pH 7.0)
2, 1-OH-tacrine	Dog liver micro-somes	Protein precipitation with ACN	Zorbax Rx-C_{18} (150 × 4.6 mm, 5 µm) Metabolite separation: Gradient, 4% ACN : 96% D_2O (0.01% TFA) increasing to 16% ACN over 40 min
2, *N*-butyramide-tacrine (BTHA)	Mouse plasma and brain homo-genate	Protein precipitation with methanol	Shim-Pack C_{18} (150 × 6 mm, 5 µm) Gradient, 35% methanol : 65% 0.1 M phosphate buffer (pH 6.1) held for 5 min increasing to 70% methanol

	Detection	Calibration range[a]	LOD/LOQ[a]	Recovery[a]	Reference
	TSP/MS, UV (325 nm), ^{1}H-NMR	nd	nd	Urine: 60% Plasma: 70-90%	Pool *et al.*, 1997
	Fluorescence (330 and 360 excitation and emission)	2, 1-OH-tacrine, 2-OH-tacrine: 0.5-50 ng/mL 4-OH-tacrine: 0.5–25 ng/mL	LOQ: 0.3 ng/mL	67% 2-OH-tacrine: 39%	Aymard *et al.*, 1998
	Fluorescence (330 and 365 excitation and emission)	2-OH-tacrine and 4-OH-tacrine Plasma: 1–12 ng/mL Urine: 24–416 ng/mL 2 and 1-OH-tacrine Plasma: 5-50 ng/mL Urine: 95-1663 ng/mL	LOD Plasma: 2-OH-tacrine and 4-OH-tacrine: 0.5 nM 2 and 1-OH-tacrine: 2nM Urine: 2-OH-tacrine and 4-OH-tacrine: 60nM 1-OH-tacrine: 30nM 2: 80nM	Plasma: 84 – 105% Urine: 64 – 100%	Hansen *et al.*, 1998
	Fluorescence (330 and 365 excitation and emission)	0–122 ng/mL	LOD: 0.4 ng/mL LOQ: 1.0 ng/mL	>87%	Chollet *et al.*, 2000
	UV (320 nm), ESI-MS, ^{1}H-NMR	nd	nd	nd	Bao *et al.*, 2002
	UV (240 nm)	BTHA: 0.25–20 µg/mL 2: 0.025–20 µg/mL	LOD BTHA: 200 ng/mL 2: 20 ng/mL	Plasma: BTHA: 91% 2: 71% Brain: BTHA: 69% 2: 73%	Jiang *et al.*, 2003

Table 2.3 Summary of the most relevant methods developed for the determination of donepezil (**3**) and its metabolites in different biological matrices. a if not stated otherwise the given values refer to all listed substances and conditions. Abbreviations are listed in the abbreviation list.

Substance	Matrix	Sample preparation	Separation
Donepezil (**3**), enantiomers	Rat plasma	Protein precipitation with ACN	Chiral column for racemate resolution Ultron ES-OVM (150 × 4.6 mm, 5 µm) 12.5% ACN : 87.5% 20 mM phosphate buffer (pH 7.2)
3, enantiomers	Human plasma	LLE (isopropanol-*n*-hexane)	Chiral column Bioptick AV-1 (150 × 2.1 mm, 5 µm) 25% methanol : 75% 10mM formic acid
3	Human plasma	LLE (isopropanol-*n*-hexane and back extracted with HCl)	STR ODS-II C_{18} (150 × 4.6 mm, 5 µm) 40% ACN : 59.5 % 0.02 M phosphate buffer (pH 4.6) : 0.5% perchloric acid (6 M)
3	Human plasma	LLE (ethyl acetate)	Aquasil C_{18} (150 × 2.1 mm, 5 µm) 70% methanol : 10% ACN : 20% water (1% formic acid)
3, enantiomers	Tablets and rat plasma	Plasma: protein precipitation with ACN Tablets: methanol extraction	Chiral column Chiralcel OD (250 × 4.6 mm, 10 µm) 87% *n*-hexane : 12.9% isopropanol : 0.1% triethylamine
3	Rat and human plasma, microdialysate samples (blood and brain)	LLE (isopropanol-*n*-hexane	Develosil Combi C_{30} (50 × 4.6 mm, 5 µm) Plasma samples: 27% ACN : 73% 25 mM citric acid/50 mM NaH_2PO_4 (pH 6.0) containing 3.5 mM sodium 1: octanesulfonate for Microdialysate samples: 17% ACN : 3% methanol : 80% water (0.01% acetic acid)

	Detection	Calibration range[a]	LOD/LOQ[a]	Recovery[a]	Reference
	Fluorescence (318 and 390 nm excitation and emission)	5–500 ng/mL	LOD: 1 ng/mL	>99%	Haginaka and Seyana, 1992
	ESI-MS/MS	0.021–50.0 ng/mL	LOQ: 0.02 ng/mL	>90%	Matsui *et al.*, 1999
	UV (315 nm)	3–90 ng/mL	LOQ: 3 ng/mL	92.7 – 94.6%	Yasui-Furukori *et al.*, 2002
	ESI-MS/MS	0.1–20 ng/mL	LLOQ: 0.1 ng/mL	>65%	Xie *et al,* 2006
	UV (268 nm)	0.05–2 μg/mL	LOQ: 0.05 μg/mL LOD: 20 ng/mL	93%	Radwan *et al,* 2006
	Fluorescence (325 and 390 excitation and emission)	Rat plasma: 5–500 nM Microdialysates: 10–500 nM Human plasma: 1–50 nM	LOD: Human plasma: 0.2 ng/mL rat plasma: 1.0 ng/mL microdialysate: 2.1 ng/g	Rat plasma: 91% Human plasma: 101.7%	Nakashima *et al.* 2006

Table 2.3 cont.

Substance	Matrix	Sample preparation	Separation
3	Dog plasma	Online sample pretreatment	Symmetry C_{18} (100 × 2.1 mm, 3.5 µm) Gradient, 10 % ACN : 90% H_2O : 1% TFA (A) 90% ACN-1% TFA (B)
3	Human plasma	LLE (*n*-hexane)	YMC Pack ODS-A (50 × 4.0 mm, 5 µm) 82% ACN : 18% 10mM ammonium acetate (pH 5.0)
3	Human plasma	LLE (isopropanol-*n*-hexane)	FASS-CE Uncoated fused-silica capillary (50.2 cm and 50 µm id) 60 mM tris (pH 4.0) -40 mM sodium octanesulfate : 0.01% polyvinyl alcohol.
3	Human plasma	SPE (Waters Oasis HLB cartridges)	Betabasic C_8 (100 × 4.6 mm, 5 µm) 90% methanol : 9.97% H_2O : 0.03% formic acid
3, 6-*O*-desmethyl donepezil (6-ODD)	Human plasma	SPE (Waters Oasis HLB cartridges)	Novapack C_{18} (150 × 3.6 mm, 4 µm) 20% methanol : 17% ACN-63% 20mM ammonium acetate (pH 3.43) : 0.2% formic acid

	Detection	Calibration range[a]	LOD/LOQ[a]	Recovery[a]	Reference
	ESI-MS	0.01–20 ng/mL	LOD: 1pg/mL LOQ: 10pg/mL	97.0 – 100.9%	Asakawa *et al.*, 2007
	ESI-MS/MS	0.1–100 ng/mL	LOQ: 0.1 ng/mL	57 – 92%	Apostolou *et al.*, 2007
	UV (200 nm)	1–50 ng/mL	LOD: 0.1 ng/mL LOQ: 0.5 ng/mL	94%	Yeh *et al.*, 2008
	ESI-MS/MS	0.15–50 ng/mL	LOQ: 0.15 ng/mL	>89%	Shah *et al.*,2008
	ESI-MS/MS	3: 0.10–50.0 ng/mL 6-ODD: 0.02–10.0 ng/mL	LLOQ: 3: 0.10 ng/mL 6-ODD: 0.02 ng/mL	3: 58% 6-ODD: 66%	Patel *et al.*, 2008

Table 2.4 Summary of the most relevant methods developed for the determination of rivastigmine (**4**) and its metabolites in different biological matrices. a if not stated otherwise the given values refer to all listed substances and conditions. Abbreviations are listed in the abbreviation list.

Substance	Matrix	Sample preparation	Separation
Rivastigmine (**4**), NAP 226-90	Human plasma	LLE (methyl-tert-butyl ether)	Purosphere Star C_{18} (55 × 2.0 mm, 3 µm) 55 % methanol : 45% 20 mM ammonium acetate
4	Dog plasma	Solid phase microextraction	GC-MS, HP-5MS capillary column (30m × 0.25mm id × 0.25 µm film)
4, NAP 226-90	Rat brain and plasma	LLE (ethyl acetate)	Nucleosil C_{18} (125 × 2.0 mm, 5 µm) 80% ACN : 20% H_2O : 0.1 % formic acid
4, NAP 226-90	Human plasma	Protein precipitation with methanol	Gemini C_{18} (150 × 2.0 mm, 5 µm) Gradient, 50% Methanol : 50% 10 mM ammonium hydroxide increasing to 95% methanol in 0.1min, kept for 7min
4	Human plasma	SPE (Waters Oasis HLB cartridges)	Betabasic C_8 (100 × 4.6 mm, 5 µm) 70% ACN : 30 % H_2O-0.1% formic acid
4, NAP 226-90, **5** (details given in table 5)	Human plasma	LLE (diethylether)	MEKC Uncoated fused-silica capillary (30.2 cm, effective length 20 cm, 50 µm id) 25 mM Tris buffer (pH 5.0) : 160 mM SOS-20% ACN : 0.01% poly(vinylpyrrolidone)

	Detection	Calibration range[a]	LOD/LOQ[a]	Recovery[a]	Reference
	APCI-MS/MS	0.20–30.0 ng/mL	LOQ: 0.2 ng/mL	**4**: 115% NAP 226-90: 88%	Pommier *et al.*, 2003.
	EI-MS	0.20–80.0 ng/mL	LOQ: 0.2 ng/mL	nd	Sha *et al.*, 2004
	ESI-MS/MS	**4**: 10–100 pmol/mL in plasma, pmol/g in brain	LOQ Plasma: **4**: 0.5 pmol/mL NAP 226-90: 1 pmol/mL Brain: **4**: 2.5 pmol/g NAP 226-90: 5 pmol/g	**4**: 93% NAP 226-90: 95%	Enz *et al.*, 2004
	ESI-MS/MS	**4**: 0.25–50.0 ng/mL NAP 226-90: 0.50-25 ng/mL	LOQ **4**: 0.25 ng/mL NAP 226-90: 0.50 ng/mL	**4**: 71% NAP 226-90: 86%	Frankfort *et al.*, 2006
	ESI-MS/MS	0.20–20.0 ng/mL	LOQ: 0.2 ng/mL	86%	Bhatt *et al.*,2007
	UV (200 or 214 nm)	0.5–50 ng/mL	LOD NAP 226-90: 0.125 ng/mL LOQ NAP 226-90: 0.5 ng/mL	**4**: >90% NAP 226-90: >91%	Hsieh *et al.*, 2009

Table 2.5 Summary of the most relevant methods developed for the determination of galantamine (**5**) and its metabolites in different biological matrices. a if not stated otherwise the given values refer to all listed substances and conditions. Abbreviations are listed in the abbreviation list.

Substance	Matrix	Sample preparation	Separation
Galantamine (**5**)	Human serum	Protein precipitation with trichloroacetic acid followed by LLE (dichloromethane)	Chrompack Microspher Si (100 × 4.6 mm, 3 µm) 50% DCM : 50% *n*-hexane : 0.25% ethanolamine
5, Epigalantamine, Galantaminone	Human plasma and urine	Plasma: protein precipitation with trichloroacetic acid followed by LLE (Chloroform) Urine: no precipitation step	Hibar-LiChrosorb C_8 (125 × 4 mm, 5 µm) 40% Methanol : 60 % water (5mM dibutylamine (pH 7)
5, Epigalantamine	Mouse plasma and tissues	Protein precipitation with triclhoroacetic acid, followed by LLE (chloroform)	Inertsil C_8 (250 × 4 mm, 5 µm) 12% ACN : 85% water : 3% tetrahydrofuran: 0.1% dibutylamine (pH 7)
5	Heparinised human plasma	LLE (toluene)	Symmetry shield C_{18} (50 × 4.6 mm, 3.5 µm) 15 % ACN : 85% 10 mM ammonium acetate
5, *O*-desmethyl galantamine, *N*-desmethyl galantamine, *N*-oxide-galantamine, Epigalantamine	Rat plasma, liver, brain and pituitary gland	SPE (Water Oasis MCX cartridges)	Discovery HS F5 C_{18} (150 × 4.6 mm, 5 µm) Gradient, 15 % ACN : 85% 5 mM ammonium acetate (pH 6.8) increasing to 25% ACN in 4 min, held for 30 min
5, Rivastigmine, NAP 226-90	Human plasma	LLE (diethylether)	MEKC Uncoated fused-silica capillary (30.2 cm, effective length 20 cm, 50 µm id) 25mM Tris buffer (pH 5.0), 160mM SOS-20% ACN-0.01% polyvinylpyrrolidone

	Detection	Calibration range[a]	LOD/LOQ[a]	Recovery[a]	Reference
	UV (235 nm)	5–100 ng/mL	LOD: 5 ng/mL	100.2%	Claessens *et al.*, 1983
	UV (280 nm)	0.1–10 µg/mL	LOD: 50 ng/mL	>95%	Tencheva *et al.*, 1987
	Fluorescence (290 and 320 nm, excitation and emission)	nd	LOD: 5ng/mL	>75%	Bickel *et al.*, 1991
	ESI-MS/MS	1.0–500 ng/mL	LOQ: 1 ng/mL	92.9 – 97.6%	Verhaeghe *et al.*, 2003
	Fluorescence (280 and 310 nm excitation and emission), ESI-MS	23–5635 ng/mL	LOD Plasma: **5**: 8.6 ng/mL *O*-desmethyl-galantamine: 52 ng/mL *N*-desmethyl-galantamine and epigalantamine: 20 ng/mL	**5**: 81% 0-desmethyl galantamine: 57.8% *N*-desmethyl galantamine: 49.7% Epigalantamine: 81.3%	Malakova *et al,* 2007
	UV (200 or 214 nm)	1.0–120.0 ng/mL	LOD 0.25 ng/mL LOQ 1.0 ng/mL	85 – 100.1%	Hsieh *et al.*, 2009

Table 2.6 Summary of the most relevant methods developed for the determination of huperzine (**6**) and its metabolites in different biological matrices. a if not stated otherwise the given values refer to all listed substances and conditions. Abbreviations are listed in the abbreviation list.

Substance	Matrix	Sample preparation	Separation
Huperzine A (**6**)	Dog plasma	LLE (*n*-hexane-dichloromethane-2-propanol)	Nucleosil C_{18} (50 × 4.6 mm, 5 µm) 35% ACN : 40% Methanol : 25% 10 mM ammonium acetate
6, ZT1 (rZT1)	Rat blood	LLE (ethyl acetate-isopropanol), ZT1 reduced to its stable derivative rZT1 using $NaBH_4$	Supelcosil C_{18} (100 × 3 mm, 5 µm) Gradient (A) 2% methanol : 98% H_2O : 0.05% ammonium formate (B) 90% methanol : 10% H_2O-0.05% ammonium formate
6, ZT1	Rat plasma	LLE (chloroform-isopropanol)	Nucleosil ODS C_{18} (250 × 4.6 mm, 5 µm) 30% methanol : 70% 10 mM ammonium acetate
6	Human plasma	LLE (ethyl acetate)	Diamonsil C_{18} (150 × 4.6 mm, 5 µm) 60% Methanol : 40% H_2O : 0.1 % formic acid
6	Human serum	LLE (dichloromethane) Labeling with rhodamine B isothiocyanate	CE Uncoated silica capillary (37 cm, 75µm id) 20 mM phosphate buffer (pH 9.5) : 10% methanol
6	Rat plasma	LLE (chloroform)	Kromasil ODS C_8 (150 × 4.6mm x 5 µm) 45% methanol : 55% H_2O : 0.05% triethanol amine
6	Rat CSF and plasma	Plasma: LLE (chloroform)	CSF: Elite ODS C_{18} (250 × 4.6mm, 10 µm) 60% methanol : 40% H_2O : 0.05% triethanol amine Plasma: cf. Yue *et al.*, 2007a
6	Rat plasma and CSF	Impurity trapping in a precolumn	Zorbax SB C_{18} (150 × 3.1 mm, 3.5µm) 30% ACN : 70% 5 mM ammonium formate-0.03% diethylamine

	Detection	Calibration range[a]	LOD/LOQ[a]	Recovery[a]	Reference
	ESI-MS/MS	0.05–20 ng/mL	LOD: 0.01 ng/mL	73 – 84%	Wang *et al.*, 2004
	ESI-MS/MS	6: 0.51–125 ng/mL rZT1 (ZT1): 0.12–30 ng/mL	LLOQ: 6: 0.51 ng/mL rZT1: 0.12 ng/mL	>88%	Li *et al.*, 2004
	UV (313 nm)	6: 5–1210 ng/mL ZT1: 7–656 ng/mL	LOQ 6: 5 ng/mL ZT1: 7 ng/mL	6: 99 – 102% ZT1: 90 – 108%	Wei *et al.*, 2006
	ESI-MS/MS	0.126–25.2 ng/mL	LOD 0.064 ng/mL LOQ 0.126 ng/mL	83.4%	Li *et al.*, 2007
	Laser induced fluorescence (532 nm), MALDI-TOF-MS	nd	LOD CE-LIF 4 nM MALDI-TOF 20 nM	nd	Hameda *et al.*, 2007
	Fluorescence (310 and 370 excitation and emission)	2.5–250 ng/mL	LOD: 2.5 ng/mL LOQ: 6.25 ng/mL	101.9-108.9%	Yue *et al.*, 2007a
	Fluorescence (310 and 370 excitation and emission)	nd	LOD: 132 ng/mL	nd	Yue *et al.*, 2007b
	ESI-MS	0.5–500 ng/mL	LOD 0.25 ng/mL LOQ 0.50 ng/mL	> 94%	Pan *et al.*, 2009

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