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

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Chapter 1

General introduction

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Alzheimer's disease was first reported by the German neuropathologist Alois Alzheimer in 1906, which explains the name of the disease. Alzheimer's disease (AD) is an incurable neurodegenerative disease that at present affects more than 25 million people worldwide, mostly people over 65 years old (Castellani *et al.*, 2010). In the Netherlands, the number of people suffering from AD was 170,000 in the year 2000 and is estimated to be almost 500,000 in 2050, which for a small country corresponds to a high number of cases (Kümpers *et al.*, 2006).

AD is the seventh leading cause of death in the United States of America (USA) and is virtually tied with the sixth leading cause of death diabetes (Alzheimer's association, 2010). In the USA, AD prevalence was estimated to five million persons in 2007 and is projected to increase to thirteen million in 2050. Furthermore, the number of AD patients and other dementias in the USA is increasing every year due to the growth of the older population (Alzheimer's association, 2010). This number will continue to increase and can escalate rapidly in the coming years as the "baby boom generation" ages.

Many studies focus on improving the quality of life for AD patients. These studies are partly based on different hypotheses on the causes of AD, that are the cholinergic, the amyloid, the tau and the excitotoxicity hypothesis (degenerative cascade).

The cholinergic hypothesis states that AD is caused by a decrease of the cholinergic neurotransmission which in turn is caused by a shortage of acetylcholine, an important chemical messenger for learning- and memory- processes. The degradation of acetylcholine into choline and acetic acid is catalyzed by the enzyme acetylcholinesterase (AChE) (Auterhoff and Hamacher) that is present in the synapse between the nerves. Various anti-AD drugs that are available in the market inhibit AChE and thereby slow down the worsening of the symptoms caused by AD. By inhibiting AChE, the level of acetylcholine rises resulting in an increased cognition between the neurons (Sugimoto, 2008).

The amyloid hypothesis was proposed in the early 90s, and its essence is that the increased production or decreased clearance of Aβ peptides causes the disease. Accumulation of the Aβ40 and Aβ42 peptides results in aggregation and formation of insoluble plaques, which trigger a cascade of deleterious changes, resulting in neuronal death and thus causing AD. Overtime this hypothesis has undergone alterations due to facts that did not correlated well with some findings, as cases of AD patients with severely impaired memory showed no plaques at post-mortem analysis (Pimplikar, 2009). Furthermore recent advances in neuroimaging techniques in vivo have shown the presence of robust plaques in otherwise cognitively normal people (Villemagne *et al.*, 2008). These observations have led to thinking that perhaps the insoluble plaques does not trigger the pathological events, and may be benign or even protective in nature (Pimplikar, 2009). There are also observations that Aβ accumulates intracellularly in mouse models of AD and in human AD brains and could contribute to disease progression (LaFerla *et al.*, 2007). Although the amyloid hypothesis remains the best defined and more widely accepted view, the evidence that Aβ causes the disease is not as strong as the

supporters would like to believe and for those who believe the amyloid hypothesis to be completely wrong do not have data to support such a claim (Pimplikar, 2009).

The tau hypothesis is based on the concept that hyperphosphorylation of the tau protein constitutes a final common pathway in AD. These hyperphosphorylated tau oligomers exert pathological effects, triggering neurotoxic actions that affect the normal interaction patterns of the neuronal cytoskeleton, which seems to correlate with cognitive impairment (Maccioni *et al.*, 2010).

The excitotoxicity hypothesis has been extensively investigated for a variety of neurodegenerative diseases. Excitotoxicity is a neuron degeneration caused by excessive exposure to excitatory amino acids (EAA). L-glutamate is an EAA and has been identified as the principal transmitter mediating fast excitatory synaptic responses in the vertebrate central nervous system (CNS) (Collingridge and Lester, 1989). Glutamate receptors are divided on the basis of their mode of action and pharmacological properties into two major subdivisions: ionotropic channel receptors (iGluR) and metabotropic G-protein coupled receptors (mGluR). iGluRs are characterized by their selective affinity for specific agonists: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid and kainic acid (Hynd *et al.*, 2004). NMDA receptors constitute a major class of Glu receptors in the mammalian CNS (MacDonald *et al.*, 1989), and can mediate post-synaptic Ca^{2+} influx. Koh and Choi performed in vitro experiments and showed that the accumulation of radio labeled Ca^{2+} correlates closely with the degenerative process, suggesting that the toxicity is principally mediated by Ca^{2+} influx probably via NMDA receptors (Koh and Choi, 1991). Memantine, the drug targeting this mechanism, acts as a NMDA antagonist. It improves the signal-to-noise ratio of glutamatergic transmission and protects cortical neurons from the toxic effects of chronic over exposition to glutamate (Parson *et al.*, 2007).

AD is one of the human disorders that have triggered the largest number of hypotheses to explain its pathogenesis, probably by the fact that no cure has yet been found for this devastating disease. Other hypotheses which are not outlined here, such as oxidative stress (Praticò, 2008) and metal hypothesis (Bush and Tanzi, 2008) are also under investigation to find new treatment possibilities and biomarkers for an early recognition of AD (Khan and Alkon, 2010, Nichols *et al.*, 2006, Trojanowski *et al.*, 2010). Most of the anti-AD drugs currently available on the market (galantamine, donepezil and rivastigmine) are based on the cholinergic hypothesis. Memantine is the only drug targeting NMDA receptors. Further on, it is the only substance registered for the treatment of moderate to severe AD patients (Parsons *et al.*, 2007).

Enormous amounts of potential drug candidates are discovered and synthesized in today's drug discovery campaigns (Ali *et al.*, 2010, Bolognesi *et al.*, 2003, Korabecny *et al.*, 2010). Scientists search for compounds which present higher potencies with new modes of action and better toleration. Huperzine A, an alkaloid isolated from Huperzia serrata, can be named as a recent successful example in the context of AD. Huperzine A is entering phase II clinical trials in the USA and is already being used for AD treatment in China, since 1996 (Ma *et al.*, 2007).

The development of drug candidates consists of four distinct stages: 1) drug discovery, 2) preclinical development; 3) clinical development; and 4) manufacturing. In Table 1.1 a brief

summary of the four stages of drug development is shown. The drug discovery stage is used to investigate thousands of compounds by employing fast screening approaches, leading to a generation of lead compounds and then narrowing down the selection through targeted synthesis and selective screening (lead optimization) (Lee and Kerns, 1999). This leads to a final selection of the most viable therapeutic candidates that are further characterized. In the preclinical development stage, the characterization of the new drug substance is initiated, which includes preliminary information on stability, preparation, and control for manufacturing purposes (Kazakevich and LoBrutto, 2007). Stability of the new drug substance and drug products for at least 6 months is required. Appropriate data confirming the stereochemical homogeneity of the drug substance during stability studies, validation of analytical methods, and manufacture of the drug products are also required. Also at this stage appropriate bioanalytical methods are developed for the evaluation of pharmacokinetics, typically a series of studies focusing on absorption, distribution, metabolism and excretion (ADME) in animal species.

Nowadays, most pharmaceutical companies are evaluating compounds for drug-like properties, such as solubility, lipophilicity and stability, at early stages in the discovery process, which allows identification of potential development challenges and thus the selection of the best candidates for lead optimization (Chen *et al.*, 2006). The clinical development stage is constituted of three distinct phases (I-III). Each phase involves process scale-up, pharmacokinetics, drug delivery, and drug safety activities. In the manufacturing stage, the capability for the production of large quantities of synthesized drug substance or drug product is ensured. Once formulated, the drug is packaged and prepared for distribution to pharmacy stores. Manufacturing processes and facilities undergo a preapproval regulatory review and periodic inspections once production is in progress. Analytical procedures and information databases are formalized into standard operating procedures (SOPs) and product specifications. This information and technology are formally transferred for routine monitoring and release by quality control (QC) scientists. During the manufacturing stage, comparisons are made to other drug products in the same category, including stability, bioavailability, and purity. Thus, comparative information is of interest during the transition from exclusive patent-protected drugs to the open generic market (Lee and Kerns, 1999).

The stability property plays an important role in drug development and is one of the main topics of this thesis. Stability tests are performed based on guidelines issued by the International Conference on Harmonisation (ICH). The ICH is a unique project that connects the regulatory authorities of Europe, Japan and the USA as well as the experts from the pharmaceutical industry in these three regions. The main purpose is to create a more economical use of human, animal and material resources in quality, safety, efficacy, and multidisciplinary, or so called 'Q', 'S', 'E' and 'M' testing guidelines (ICH, 2010). The six co-sponsors of ICH are: (1) the Commission of the European Union; (2) the European Federation of Pharmaceutical Industries' Associations (EFPIA); (3) the Ministry of Health and Welfare, Japan (MHW); (4) the Japan Pharmaceutical Manufacturers Association (JPMA); (5) the US Food and Drug Administration (FDA); (6) the Pharmaceutical Research and Manufacturers of America (PhRMA).

Table 1.1 The four stages of drug development (Lee and Kerns, 1999).

Development stage	Milestone	Analysis emphasis	LC/MS analysis activities
Drug discovery	Lead candidate	Screening	Protein identification; natural products identification; metabolic stability profiles; molecular weight determination for combinatorial/ medicinal chemistry support.
Preclinical development	IND/CTA filing	Evaluation	Impurity, degradant and metabolite identification
Clinical development	NDA/MAA filing	Registration	Quantitative bioanalysis; structure identification
Manu-facturing	Sales	Compliance	Impurity and degradant identification

IND: investigational new drug/CTA: clinical trial application
NDA: new drug application / MAA: marketing authorization application
IND and NDA are required documents filed in the USA.
CTA and MAA are required documents in Europe.

The International Federation of Pharmaceutical Manufacturers Associations (IFPMA) participates as an 'umbrella' organization for the pharmaceutical industry, providing the ICH secretariat. The World Health Organization (WHO), European Free Trade Association (EFTA) and Canada (represented by Health Canada) serve as observers to the ICH (Muller *et al.*, 1999).

ICH requires that a drug substance has to be tested under different stress conditions. It is suggested that stress testing includes the effect of pH, temperature, humidity, light and oxidizing agents. These tests are included in the guidelines Q1A (R2) "Stability Testing of New Drugs Substances and Products" and Q1B "Stability testing: Photostability Testing of New Drug Substances and Products" (ICH, 2003, ICH, 1996b).

Although the process to release a drug formulation needs to be in conformity with ICH norms, for many authors the guidelines do not provide detailed enough information with regard to: how to perform the stability assays (Brower *et al.*, 1998, Christensen *et al.*, 2000, Thatcher *et al.*, 2001). Therefore, many reviews have been published to clarify the statements in the ICH guidelines (Baertschi *et al.*, 2010, Bakshi and Singh, 2002, Ho and Chen, 1996, Ho and Chen, 1997). In these reviews the stability assays are explained with more details. The hydrolytic degradations are performed starting from 0.1 M of NaCl/NaOH under reflux. If no degradation is seen the drug should be refluxed under higher strength of acid/alkali and under longer duration. In a similar manner degradation under neutral conditions should be performed.

To test for oxidation a concentration of 3% to 30% of hydrogen peroxide should be use. The photolytic studies should be carried out by exposure to light, using either a combination of cool white and ultraviolet fluorescent lamps, or one among the xenon and metal halide lamps. Samples should be submitted to wavelengths above 310 nm. This condition mimics the real environment of UV exposition to sun light, due to the fact that clouds and dust particles absorbs the lower wavelengths in the atmosphere (Anderson *et al.*, 1991).

In order to generate high-quality data of stability studies, a specific, selective, robust and sensitive stability-indicating assay needs to be developed and validated. An ideal assay should allow detection of degradation peaks equivalent to 0.1% of the parent peak. Chromatography is the technique that is mainly used for stability studies. Other than separation of multiple components, the advantage of chromatographic methods is that it possesses good accuracy and sensitivity for even small quantities of degradation products produced (Bakshi and Singh, 2002). Various chromatographic methods that have been used for stability assays include: thin-layer chromatography (TLC) (Bebawy *et al.*, 2003, Mostafa, 2010, Youssef, 2005), high-performance thi*n*-layer chromatography (HPTLC) (Kaul *et al.*, 2004, Vadera *et al.*, 2007, Venkatachalam and Chatterjee, 2007), gas chromatography (GC) (Avramides, 2005, Lima *et al.*, 2005), HPLC (Fu *et al.*, 2010, Kasawar and Farooqui, 2010, Naguib and Abdelkawy, 2010, Xiong *et al.*, 2009), and capillary electrophoresis (CE) (Alnajjar *et al.*, 2007, Baalbaki *et al.*, 2005, Hamdam *et al.*, 2010).

More recently, huge attention is paid to genotoxic and carcinogenic effects of drugs, their degradation products and impurities. Genotoxicity tests can be defined as in vitro and in vivo tests designed to detect compounds that induce genetic damage by various mechanisms. Carcinogenicity tests are done mostly with drug substances that are used continuously for 6 months or longer (ICH, 1995b). Guidelines for testing genotoxic and carcinogenic effects can be found in the guideline S2(R1): "Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use"(ICH, 2008b), and in S1A, "Guideline on the Need for Carcinogenicity Studies of Pharmaceuticals" (ICH, 1995a), S1B: "Testing for Carcinogenicity of Pharmaceuticals"(ICH, 1997a) and S1C(R2): "Dose Selection for Carcinogenicity Studies of Pharmaceuticals"(ICH, 2008a). Performance of genotoxicity tests has improved over the years and the chances of failing to detect true genotoxic activity in a standard or extended battery of tests are small. Nevertheless, the area is still open for more sensitive, more practical, more expeditious, and more economical techniques for detection of genotoxic compounds (ICH, 1997b).

In parallel with toxicity tests, biochemical characterization of degradation compounds towards a specific target, in this case AChE, would be interesting to perform as degradation products could possibly provide new drug candidates for further investigation (Pleuvry, 2006, Tsuikiura *et al.*, 1973). It is observed that not many stability studies pay attention to the biochemical characterization of the formed degradation products.

This thesis provides stability assays based on the ICH guidelines of the anti-AD drugs tacrine, huperzine A, and galantamine. The three drugs have been subjected to various stress testing conditions (effect of pH, temperature, humidity, light and oxidizing agents) in order to study

degradation and degradation kinetics. In addition, structure elucidation of degradation products and bioactivity testing of the formed substances against AChE has been performed.

In Chapter 2, an overview of the current literature is presented regarding degradation studies, metabolism as well as bioanalysis of the anti-AD drugs that were used in the past and the ones that are in current use. In this chapter, more detailed information about these drugs is given, such as stability, methodologies used for their detection and quantification in different biological matrices, and methods related to sample pretreatment. Moreover, the metabolism pathways of these drugs are also illustrated. The chapter is concluded by an overview about the most frequently used sample pretreatment procedures and perspectives of future applications.

In Chapter 3, a stability study and an on-line AChE fluorescence based high-resolution screening (HRS) assay of the anti-AD drug tacrine is presented. The concept of HRS based assays is the integration of analytical separation with biochemical detection in an on-line setup. The effluent of the analytical column is first mixed with the protein under investigation (AChE) and subsequently, a substrate or a molecular probe is added. If no bioactive compound is eluting from the analytical column, the enzyme stays active and is for example continuously generating a fluorescent product from a non-fluorescent substrate. If a bioactive substance elutes from the analytical column and binds to the enzyme, the enzymatic activity is reduced, thereby giving a negative peak in the biochemical detection system. The detection methods used for HRS assays usually are based on ultraviolet (UV) (Ingkaninan *et al.*, 2000), fluorescence (Marques *et al.*, 2010, Rhee *et al.*, 2003), (laser induced) fluorescence (Heus *et al.*, 2010) and mass spectrometry (MS) based (de Jong *et al.*, 2006) detection. Fluorescence and MS based detection are most frequently applied because of their better sensitivity compared to UV detection.

The assay formats depend on the drug target class which is incorporated (Kool *et al.*, 2010). Some successful examples include: protein kinases (Falck *et al.*, 2010), phosphatases (Schenk *et al.*, 2003), proteases (Schebb *et al.*, 2009) and drug metabolizing enzymes (Liempd *et al.*, 2005). In this thesis, a HRS system was applied for the screening of degradation products of tacrine in an on-line manner and to test their biological activity against AChE.

Stability assays showed that tacrine was basically stable under acidic and basic conditions as well as at elevated temperatures. Under hydrogen peroxide treatment, tacrine was degraded to a series of mono- di- and tri- hydroxylated compounds. These products were characterized chemically and biochemically and a comparison study with the metabolic pattern generated by P450 microsomal incubation was carried out. It was observed that some of the degradation products formed by P450 transformation were also formed under treatment with hydrogen peroxide. This was especially interesting as these compounds could be easily formed in batch amounts, thereby generating sufficient material for NMR analysis.

In Chapter 4, the stability of the anti-Alzheimer's drug Huperzine A was studied. Huperzine A was found to be stable under all conditions, except under light exposure. Under irradiation with wavelengths above 310 nm, huperzine A showed transformation into an unknown substance, which is a new photoisomer of huperzine A, named photohuperzine A. The initial structure

elucidation step for the formed compound was triggered by the observation that the degradation product lost its UV absorption maximum at 308 nm (typical 2-pyridone absorption). MSn experiments of the product did not give any insights into the structure of the degradation product, basically showing similar mass spectra to huperzine A. Hence, the compound was isolated and its structure was fully elucidated by using diverse NMR techniques (^{1}H-NMR, ^{13}C-NMR, COSY, HMBC, HSQC and NOESY), revealing a photoisomerization of the 2-pyridone ring of huperzine A. Interestingly, the compound showed a 100 times lower bioactivity against AChE than huperzine A itself, which proved that the planarity of the pyridone ring is important in the activity against AChE. Additionally, the kinetics of the photodegradation was investigated.

In Chapter 5, a validated stability-indicating method for the anti-AD drug galantamine was developed. Galantamine was found to be stable only under alkaline conditions and at elevated temperatures. The degradation kinetics and the structure elucidation of the degradation compounds found under acidic, oxidative and photolytic conditions were achieved by MS and NMR (^{1}H-NMR, ^{13}C-NMR, COSY, HMBC, HSQC and NOESY) techniques. The isolated degradation products showed lower activity than the parent compound itself. In Chapter 6, the final conclusions and future perspectives of the work are given.

References

Ali MA, Ismail R, Choon TS, Yoon YK, Wei AC, Pandian S, Kumar RS, Osman H and Manogaran E. *Substituted spiro [2.3'] oxindolespiro [3.2"]-5, 6-dimethoxy-indane-1"-one-pyrrolidine analogue as inhibitors of acetylcholinesterase*. Bioorganic & Medicinal Chemistry Letters 2010; 20:7064-7066.

Alnajjar A, Idris AM and AbuSeada HH. *Developement of a stability-indicating capillary electrophoresis method for norfloxacin and its inactive decarboxylated degradant.* Microchemical Journal 2007; 87: 35-40.

Alzheimer'sassociation. 2010 *Alzheimer's disease facts and figures.* Alzheimer's & Dementia 2010; 6: 158-194.

Anderson NH, Johnston D, McLelland MA and Munden P. *Photostability testing of drug substances and drug products in UK pharmaceutical laboratories* Journal of Pharmaceutical and Biomedical Analysis 1991; 9: 443-449.

Avramides EJ. *Long-term stability of pure standards and stock standard solutions for the determination of pesticide residues using gas chromatography.* Journal of Chromatography A 2005; 1080: 166-176.

Baalbaki B, Cheble E, Nguema G and Fabre H. *Stability-indicating assay using capillary zone electrophoresis for an azaphenothiazine in an ointment formulation.* Analytica Chimica Acta 2005; 533: 121-125.

Baertschi SW, Alsante KM and Tonnensen HH. *A critical assessment of the ICH guideline on photostability testing of new drug substances and products (Q1B): Recommendation for revision.* Journal of Pharmaceutical Sciences 2010; 99: 2934-2940.

Bakshi M and Singh S. *Development of validated stability-indicating assay methods--critical review.* Journal of Pharmaceutical and Biomedical Analysis 2002; 28: 1011-1040.

Bebawy LI, Moustafa AA and Abo-Talib NF. *Stability-indicating methods for the determination of sumatriptan succinate.* Journal of Pharmaceutical and Biomedical Analysis 2003; 32: 1123-1133.

Bolognesi ML, Cavalli A, Andrisano V, Bartolini M, Banzi R, Antonello A, Rosini M and Melchiorre C. *Design, synthesis and biological evaluation of ambenonium derivatives as AChE inhibitors.* Il Farmaco 2003; 58: 917-928.

Brower JF, Drew HD, Julh WE and K. TL. *Quinine photochemistry: A proposed chemical actinometer to monitor UV-A exposure in photostability studies of pharmaceutical drug substances and drug products.*Pharmaceutical Forum 1998; 24: 6334-6346.

Castellani RJ, Rolston RK and Smith MA. *Alzheimer disease.* Disease-a-Month 2010; 56: 484-586.

Chen XQ, Antman MD, Gesenberg C and Gudmundsson OS. *Discovery pharmaceutics- Challenges and opportunities* The AAPS Journal 2006; 8: 402-408.

Christensen KL, Christensen JO, Frokjaer S, Langball P and Hansen LL. *Influence of temperature and storage time after light exposure on the quinine monohydrochloride chemical actinometric system* European Journal of Pharmaceutical Sciences 2000; 9: 317-321.

Collingridge GL and Lester AJ. *Excitatory amino acids receptors in the vertebrate central nervous system.* Pharmacological Reviews 1989; 40: 143-210.

de Jong CF, Derks RJ, Bruyneel B, Niessen W and Irth H. *High-performance liquid chromatography-mass spectrometry-based acetylcholinesterase assay for the screening of inhibitors in natural extracts.* Journal of Chromatography A 2006; 1112: 303-310.

Dobo KL, Greene N, Cyr MO, Caron S and Ku WW. *The application of structure-based assessment to support safety and chemistry diligence to manage genotoxic impurities in active pharmaceutical ingredients during drug development* Regulatory Toxicology and Pharmacology 2006; 44: 282-293.

Fu Q, Shou M, Chien D, Markovich R and Rustum AM. *Development and validation of a stability-indicating RP-HPLC method for assay of betamethasone and estimation of its related compounds.* Journal of Pharmaceutical and Biomedical Analysis 2010; 51: 617-625.

Hamdam II, Jaber AK and Abushoffa AM. *Development and validation of a stability indicating capillary electrophoresis method for the determination of metformin hydrochloride in tablets* Journal of Pharmaceutical and Biomedical Analysis 2010; 53: 1254-1257.

Heus F, Giera M, Kloe GEd, Iperen Dv, Buijs J, Nahar TT, Smit AB, Lingeman H, Esch IJPd, Niessen WMA, Irth H and Kool J. *Development of a microfluidic confocal fluorescence detection system for the hyphenation of nano-LC to on-line biochemical assays*. Analytical and Bioanalytical Chemistry 2010: doi: 10.1007/s00216-010-4210-x.

Ho C and Chen G-L. *Stability-indicating high-performance liquid chromatographic assay methods for drugs in pharmaceutical dosage forms: Part I.* Journal of Food and Drug Analysis 1996; 4: 271-292.

Ho C and Chen SN. *Stability-indicating high-performance chromatographic assay methods for drugs in pharmaceutical dosage forms: Part II.* Journal of Food and Drug Analysis 1997; 5: 1-24.

Hynd MR, Heat HLS and Dodd PR. *Glutamate-mediated excitotoxicity and neurodegeneration in Alzheimer's disease.* Neurochemistry International 2004; 45: 583-595.

ICH. Q3B (R2) *Impurities in New Drug Products* International Conference on Harmonisation, Geneva, June 2006. Available from: http://www.ich.org/LOB/media/MEDIA421.pdf

ICH. S1A: *Guideline on the Need for Carcinogenicity Studies of Pharmaceuticals.* International Conference on Harmonisation, Geneva, November 1995a. Available from: http://www.ich.org/LOB/media/MEDIA489.pdf

ICH. S1B: *Testing for Carcinogenicity of Pharmaceuticals.* International Conference on Harmonisation, Geneva July 1997a. Available from: http://www.ich.org/LOB/media/MEDIA490.pdf

ICH. S1C(R2): *Dose Selection for Carcinogenicity Studies of Pharmaceuticals.* International Conference on Harmonisation, Geneva, March 2008. Available from: http://www.ich.org/LOB/media/MEDIA491.pdf

ICH. S2(R1): *Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use.* International Conference on Harmonisation, Geneva, March 2008. Available from: http://www.ich.org/LOB/media/MEDIA4474.pdf

ICH. S2A: *Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals.* International Conference on Harmonisation, Geneva, July, 1995b. Available from: http://www.ich.org/LOB/media/MEDIA493.pdf

ICH. S2B: *Genotoxicity: A Standard Battery for Genotoxicity Testing for Pharmaceuticals.* International Conference on Harmonisation, Geneva, July, 1997b. Available from: http://www.ich.org/LOB/media/MEDIA494.pdf

ICH. Q1A (R2) *Stability Testing of New Drug Substances and Products.* International Conference on Harmonisation, Geneva, February 2003.Available from: http://www.ich.org/LOB/media/MEDIA419.pdf

ICH. (Q1B) *Stability testing: photostability testing of new drug substances and products.* Prodeedings of the International Conference on Harmonisation, Geneva, November 1996. Available from: http://www.ich.org/LOB/media/MEDIA412.pdf.

Ingkaninan K, Best CMd, Heijden Rvd, Hofte AJP, Karabatak B, H HI, Tjaden UR, Greef Jvd and Verpoorte R. *High-performance liquid chromatography with on-line coupled UV, mass spectrometric and biochemical detection for identification of acetylcholinesterase inhibitors from natural products* Journal of Chromatography A 2000; 872: 61-73.

Kasawar GB and Farooqui M. *Development and validation of a stability indicating RP-HPLC method for the simultaneous determination of related substances of albuterol sulfate and ipratropium bromide in nasal solution.* Journal of Pharmaceutical and Biomedical Analysis 2010; 52: 19-29.

Kaul N, Agrawal H, Paradkar AR and Mahadik KR. *The ICH guidance in practice: stress degradation studies on indinavir sulphate and development of a validated specific stability-indicating HPTLC assay method. II* Farmaco 2004; 59: 729-738.

Kazakevich Y and LoBrutto R. *HPLC for pharmaceutical scientists* 2007 Hoboken, New Jersey.

Khan TK and Alkon DL. *Early diagnostic accuracy and pathophysiologic relevance of an autopy-confirmed Alzheirmer's disease peripheral biomarker.* Neurobiology of Aging 2010; 31: 889-900.

Koh JY and Choi DW. *Selective of non-NMDA receptors does not block rapidly triggered glutamate-induced neuronal death.* Brain Research 1991; 548: 318-321.

Kool J, Lingeman H, Niessen WMA and Irth H. *High throughput screening methodologies classified for major drug target classes according to target signaling pathways.* Combinatorial Chemistry & High Throughput Screening 2010; 13: 548-561.

Korabecny J, Musilek K, Holas O, Binder J, Zemek F, Marek J, Pohanka M, Opletalova V, Dohnal V and Kuca K. *Synthesis and in vitro evaluation of N-alkyl-7-methoxytacrine hydrochlorides as potential cholinesterase inhibitors in Alzheimer disease.* Bioorganic & Medicinal Chemistry Letters 2010; 20: 6093-6095.

Kümpers S, Murb I, Hardyc B, Raak Av and Maarse H. *Integrating dementia care in England and The Netherlands: Four comparative local case studies.* Health & Place 2006; 12: 404-412.

Lee MS and Kerns EH. *LC/MS applications in drug development* Mass Spectrometry Reviews 1999; 18: 187-279.

Lima EM, Diniz DGA and R. A-FN. *Development of a gas chromatography method for the determination of isotretinoin and its degradation products in pharmaceuticals.* Journal of Pharmaceutical and Biomedical Analysis 2005; 38: 678-685.

Liu DQ, Chen TK, McGuire MA and Kord AS. *Analytical control of genotoxic impurities in the pazopanib hydrochloride manufacturing process* Journal of Pharmaceutical and Biomedical Analysis 2009; 50: 144-150.

Ma X, Tan C, Zhu D, Zhu DR and Xiao P. *Huperzine A from Huperzia species--an ethnopharmacolgical review.* Journal of Ethnopharmacology 2007; 113: 15-34.

Maccioni RB, Farías G, Morales I and Navarrete L. *The revitalized tau hypothesis on Alzheimer's disease* Archives of Medical Research 2010; 41: 226-231.

MacDonald JF, Mody I, Salter MW, Pennefather P and Schneiderman JH. *The regulation of NMDA receptors in the central nervous system.* Progress in Neuro-psychopharmacology Biological Psychiatry 1989; 13: 481-488.

Marques LA, Kool J, de Kanter F, Lingeman H, Niessen W and Irth H. *Production and on-line acetylcholinesterase bioactivity profiling of chemical and biological degradation products of tacrine.* Journal of Pharmaceutical and Biomedical Analysis 2010; 53: 609-616.

Mostafa NM. *Stability indicating method for the determination of paracetamol in its pharmaceutical preparations by TLC densitometric method* Journal of Saudi Chemical Society 2010; 14: 341-344.

Muller L, Kikuchi Y, Probst G, Schechtman L, Shimada H, Sofuni T and Tweats D. *ICH-Harmonised guidances on genotoxicity testing of pharmaceuticals: evolution, reasoning and impact* Mutation Research/Reviews in Mutation Research 1999; 436: 195-225.

Naguib IA and Abdelkawy M. *Development and validation of stability-indicating HPLC and HPTLC methods for determination of sulpiride and mebeverine hydrochloride in combination.* European Journal of Medicinal Chemistry 2010; 45: 3719-3725.

Nichols L, Pike VW, Cai L and Innis RB. *Imaging and in vivo quantitation of β-amyloid: An exemplary biomarker for Alzheimer's disease?* Biological Psychiatry 2006; 59: 940-947.

Parsons CG, Stoffler A and Danysz W. *Memantine: a NMDA receptor antagonist that improves memory by restoration of homeostasis in the glutamatergic system - too little activation is bad, too much is even worse.* Neuropharmacology 2007; 53: 699-723.

Pleuvry BJ. *Pharmacological effects of drug degradation products* Anaesthesia & Intensive Care Medicine 2006; 7: 63-65.

Rhee IK, Appels N, Luijendijk T, Irth H and Verpoorte R. *Determining acetylcholinesterase inhibitory activity in plant extracts using fluorimetric flow assay.* Phytochemical Analysis 2003; 14: 145-149.

Sugimoto H. *The new approach in development of anti-Alzheimer's disease drugs via the cholinergic hypothesis.* Chemico-biological Interactions 2008; 175: 204-208.

Thatcher SR, Mansfield RK, Miller RB, Davis CW and Baertschi SW. *Pharmaceutical photostability: A technical and practical interpretation of the ICH guideline and its application to pharmaceutical stability: Part II.* Pharmaceutical Technology 2001; 25: 50-62.

Trojanowski JQ, Vandeerstichele H, Korecka M, Clark CM, Aisen PS, Petersen RC, Blennown K, Soares H, Simon A, Lewczuk P, Dean R, Siemers E, Potter WZ, Weiner MW, Jack-Junior CR, Jagust W, Toga AW, Lee VM-Y and Shaw LM. *Update on the biomarker core of the Alzheimer's disease neuroimaging iniciative subjects.* Alzheimer's and Dementia 2010; 6: 367-377.

Tsuikiura H, Fujisawa K, Konishi M, Saito K, Numata K, Ishikawa H, Miyaki T, Tomita K and Kawaguchi H. *Aminoglycoside antibiotics.* The Journal of Antibiotics 1973; 26: 351-357.

Vadera N, Subramanian G and Musmade P. *Stability-indicating HPTLC determination of imatinib mesylate in bulk drug and pharmaceutical dosage form.* Journal of Pharmaceutical and Biomedical Analysis 2007; 43: 722-726.

Venkatachalam A and Chatterjee VS. *Stability-indicating high performance thin layer chromatography determination of paroxetine hydrochloride in bulk drug and pharmaceutical formulations.* Analytica Chimica Acta 2007; 598: 312-317.

Xiong Y, Xiao KP and Rustum AM. *Development and validation of a stability-indicating RP-HPLC method to separate low levels of dexamethasone and other related compounds from betamethasone.* Journal of Pharmaceutical and Biomedical Analysis 2009; 49: 646-654.

Youssef NF. *Stability-indicating methods for the determination of piretanide in presence of the alkaline induced degradates.* Journal of Pharmaceutical and Biomedical Analysis 2005; 39: 871-876.

Zounkova R, Kovalova L, Blaha L and Dott W. *Ecotoxicity and genotoxicity assessment of cytotoxic antineoplastic drugs and their metabolites.* Chemosphere 2010; 81: 253-260.