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PHARMACOGENETIC STUDY ON ANTIDEMENTIA DRUGS

Muriel NÖTZLI

UNIL | Université de Lausanne
Faculté de biologie et de médecine

Département de Psychiatrie

PHARMACOGENETIC STUDY ON ANTIDEMENTIA DRUGS

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par

Muriel NÖTZLI

Master en Sciences Pharmaceutiques de l'Ecole Polytechniqe Fédérale de Zurich

Jury

Prof. Lazare Benaroyo, Président
Prof. Chin B. Eap, Directeur de thèse
Prof. Panteleimon Giannakopoulos, Co-directeur
Prof. Thierry Buclin, expert
Prof. Bertrand Rochat, expert

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	Monsieur	Dr Bertrand **Rochat**

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Summary

Differences in efficacy and safety of drugs among patients are a recognized problem in pharmacotherapy. The reasons are multifactorial and, therefore, the choice of a drug and its dosage for a particular patient based on different clinical and genetic factors is suggested to improve the clinical outcome. Four drugs are currently used for the treatment of Alzheimer's disease: three acetylcholinesterase inhibitors (donepezil, galantamine, rivastigmine) and the N-methyl-D-aspartate-antagonist memantine. For these drugs, a high interindividual variability in plasma levels was observed, which might influence the response to treatment. The main objective of this thesis was to provide a better understanding of clinical and genetic factors affecting the plasma levels of antidementia drugs. Furthermore, the relationship between plasma levels, genetic variations and side effects was assessed. For this purpose, a pharmacogenetic study was conducted including 300 patients from a naturalistic clinical setting.

Analytical methods for the simultaneous measurement of antidementia drugs in plasma have been developed and validated using liquid chromatography methods coupled with mass spectrometry detection. Presently, these methods are used in the therapeutic drug monitoring service of our laboratory. The routine use of therapeutic drug monitoring for antidementia drugs cannot yet be recommended with the available data, but it may be beneficial for some patients in special clinical cases such as insufficient treatment response, side effects or drug interactions.

Donepezil and galantamine are extensively metabolized by the liver enzymes cytochromes P450 (CYP) 2D6 and 3A and are substrates of the drug transporter P-glycoprotein. The relationship of variations in genes affecting the activity of these metabolic enzymes and drug transporter (*CYP2D6*, *CYP3A, POR, NR1I2, ABCB1*) with donepezil and galantamine plasma levels was investigated. The *CYP2D6* genotype appeared to be the major genetic factor involved in the pharmacokinetics of these two drugs. Thus, CYP2D6 poor metabolizers demonstrated significantly higher drug plasma levels than extensive metabolizers. Additionally, in the donepezil study population, the frequency of side effects was significantly increased in poor metabolizers. Lower donepezil plasma levels were observed in ultra rapid metabolizers, which might expose those patients to the risk of non-response.

Memantine is mainly eliminated unchanged by the kidney, with implication of tubular secretion by renal transporters. A population pharmacokinetic model was developed to quantify the effects of clinical factors and genetic variations in renal cation transporters (*SLC22A1/2/5*, *SLC47A1*, *ABCB1*), and nuclear receptors (*NR1I2*, *NR1I3*, *PPARG*) involved in transporter expression, on memantine plasma levels. In addition to the renal function and gender, a genetic variation in the nuclear receptor Pregnane-X-Receptor (*NR1I2*) significantly affected memantine elimination.

These findings suggest that an individualized therapy approach for antidementia drugs, taking into account clinical characteristics and genetic background of a patient, might increase efficacy and safety of the treatment.

Résumé

Les différences interindividuelles dans l'efficacité et la tolérance des médicaments sont un problème connu en pharmacothérapie. Les raisons sont multiples, et le choix du médicament et de la dose, basé sur des facteurs cliniques et génétiques spécifiques au patient, peut contribuer à améliorer la réponse clinique. Quatre médicaments sont couramment utilisés dans le traitement de la maladie d'Alzheimer : trois inhibiteurs de l'acétylcholinestérase (donépézil, galantamine, rivastigmine) et un antagoniste du récepteur N-méthyl-D-aspartate, la mémantine. Une forte variabilité interindividuelle dans les taux plasmatiques de ces quatre composés a été observée, ce qui pourrait influencer la réponse au traitement. L'objectif principal de ce travail de thèse est de mieux comprendre les facteurs cliniques et génétiques influençant les taux des médicaments pro-cognitifs. En outre, des associations entre les taux, la variabilité génétique et les effets secondaires ont été recherchées. Dans ce but, 300 patients sous traitement avec un médicament pro-cognitif ont été recrutés pour une étude pharmacogénétique.

Des méthodes de dosage simultané de médicaments pro-cognitifs par chromatographie liquide couplée à la spectrométrie de masse ont été développées et validées. Ces méthodes sont actuellement utilisées dans le service de suivi thérapeutique de notre unité. Malgré le fait qu'un suivi des taux sanguins des pro-cognitifs ne puisse pas encore être recommandé en routine, un dosage peut être utile dans des cas cliniques spécifiques, comme une réponse insuffisante, une intolérance ou une interaction médicamenteuse.

Le donépézil et la galantamine sont fortement métabolisés par les cytochromes P450 (CYP) 2D6 et 3A, et sont également substrats du transporteur P-glycoprotéine. Les associations entre les polymorphismes génétiques de ces enzymes, cofacteur, récepteur nucléaire et transporteur (*CYP2D6*, *CYP3A*, *POR*, *NR1I2*, *ABCB1*) et les taux de donépézil et de galantamine ont été étudiées. Le génotype du *CYP2D6* a été montré comme le facteur génétique majeur impliqué dans la pharmacocinétique de ces deux médicaments. Ainsi, les métaboliseurs déficients du CYP2D6 ont démontré des taux plasmatiques significativement plus élevés comparé aux bons métaboliseurs. De plus, dans la population traitée avec le donépézil, la fréquence des effets secondaires était plus élevée chez les métaboliseurs déficients. Des taux plasmatiques bas ont été mesurés chez les métaboliseurs ultra-rapides traités avec le donépézil, ce qui pourrait être un facteur de risque à une non-réponse au traitement.

La mémantine est principalement éliminée sous forme inchangée par les reins, et partiellement par sécrétion tubulaire grâce à des transporteurs rénaux. Un modèle de cinétique de population a été développé pour quantifier les effets des différents facteurs cliniques et de la variabilité génétique des transporteurs rénaux (*SLC22A1/2/5*, *SLC47A1*, *ABCB1*) et des récepteurs nucléaires (*NR1I2*, *NR1I3*, *PPARG*, impliqués dans l'expression des transporteurs) sur les taux plasmatiques de mémantine. En plus de la fonction rénale et du genre, une variation génétique dans le récepteur nucléaire Pregnane-X-Receptor (*NR1I2*) a montré une influence significative sur l'élimination de la mémantine.

Ces résultats suggèrent qu'une approche thérapeutique individualisée, prenant en compte des facteurs cliniques et génétiques du patient, pourrait améliorer l'efficacité et la sécurité du traitement pro-cognitif.

Abbreviations

Aβ	Amyloid-β
AChE	Acetylcholinesterase
AChEI	Acetylcholinesterase inhibitor
ABC	ATP-binding cassette
AD	Alzheimer's disease
AE	Adverse event
APOE	Apolipoprotein E
APP	Amyloid precursor protein
AUC	Area under the curve
bp	Base pairs
BuChE	Butyrylcholinesterase
CAR	Constitutive androstane receptor
C_{av}	Mean plasma concentration at steady-state
C_{min}	Minimum plasma concentration
C_{max}	Peak plasma concentration
C_{trough}	Minimum plasma concentration
ChE	Cholinesterase
CHUV	Centre hospitalier universitaire vaudois
CL	Clearance
CNS	Central nervous system
CS	Calibration standard
CV	Coefficient of variation
CYP	Cytochrome P450
DNA	Desoxy ribonucleic acid
EM	Extensive metabolizer
EMEA	European Medicines Agency
FDA	Food and Drug Administration
GWAS	Genome-wide association studies
HPLC-MS	High performance liquid chromatography-mass spectrometry
IS	Internal standard
LLOQ	Lower limit of quantification
mRNA	Messenger ribonucleic acid
MRM	Multiple reaction monitoring
NMDA	N-methyl-D-aspartate
OCT	Organic cation transporter
PCR	Real-time polymerase chain reaction
P-gp	P-glycoprotein
PM	Poor metabolizer
POR	Cytochrome P450 oxidoreductase

PPAR	Peroxisome proliferator-activated receptor
PXR	Pregnane-X-receptor
QC	Quality control sample
RXR	Retinoid-X-receptor
SIM	Single ion monitoring
SLC	Solute carrier
SNP	Single nucleotide polymorphism
SPE	Solid phase extraction
$t_{1/2}$	Elimination half-life
t_{max}	Time to maximum peak plasma concentration
TDM	Therapeutic drug monitoring
ULOQ	Upper limit of quantification
UPLC-MS/MS	Ultra performance liquid chromatography-tandem mass spectrometry
UM	Ultrarapid metabolizer
V	Volume of distribution

Chapter 1: Introduction

1.1 Alzheimer's disease and pharmacological treatment

Over 100 years ago, Alois Alzheimer presented a case of a 51-year-old woman with mental degeneration at a congress in Germany.[1] This was the first demonstration of the disease, which was later named Alzheimer's disease (AD). He described the typical characteristics of an Alzheimer patient with memory disturbances, disorientation, aphasia, delusions and unpredictable behavior as main clinical symptoms, and the neuropathological picture with plaques and neurofibrillary tangles, which we today know as hallmarks of the disease.[1,2]

AD is the most common type of dementia, accounting for 50-60% of all cases, followed by vascular dementia, Lewy body dementia and mixed dementia (coexistence of AD and vascular dementia).[1,3] With the rapid increase of the aging population, dementia has become an important public health challenge. By 2005, the global prevalence of dementia was estimated to be as high as 24 million. The number of people affected is predicted to double every 20 years to 80 million by 2040.[4] In Switzerland, it is estimated that currently around 110 000 people suffer from dementia.[5]

As described by Alois Alzheimer, the typical features of AD are amyloid plaques caused by a gradual deposition of beta-amyloid (Aβ) peptides, and intraneuronal neurofibrillary tangles formed by aggregation of abnormally hyperphosphorylated tau proteins.[1,6] However, it is suggested that several other pathogenic mechanisms also contribute to the AD pathophysiology, such as neurovascular dysfunction, cell-cycle dysregulation, inflammatory processes, oxidative stress, and mitochondrial dysfunction.[6] The neuropathological lesions are leading to a degeneration of neurons and synapses. Thus, brain regions involved in learning and memory processes, including the temporal and frontal lobes of the cerebral cortex, are reduced in size in AD patients (Figure 1).[7]

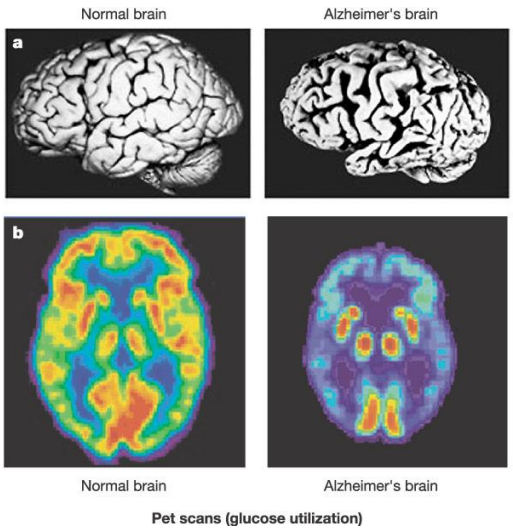

Figure 1: Alzheimer's disease results in shrinkage of brain regions involved in learning and memory (a) which is correlated with major reductions in cellular energy metabolism in living patients (red and yellow indicate high levels of glucose uptake) (b). Picture from Mattson MP, Nature, 2004;430(7000):631-9.

The current pharmacotherapy of AD is based on observed neurotransmitter disturbances, on one hand the cholinergic hypofunction and on the other hand the glutamatergic overstimulation of the postsynaptic N-methyl-D-aspartate (NMDA) receptors.[8,9] To date, the three acetylcholinesterase inhibitors donepezil, galantamine and rivastigmine are licensed in Switzerland, which inhibit the enzyme acetylcholinesterase and thereby increase the level of acetylcholine in the synaptic clefts (Figure 2a).[10,11] They are indicated for the treatment of mild to moderate AD. Moreover, the NMDA receptor antagonist memantine, acting against excessive receptor activation by glutamate, is licensed for moderate to severe AD (Figure 2b).[10,12,13] The detailed mechanism of action of each antidementia drug is described in the review article at the end of the introduction chapter (Article I, Chapter 1.3).

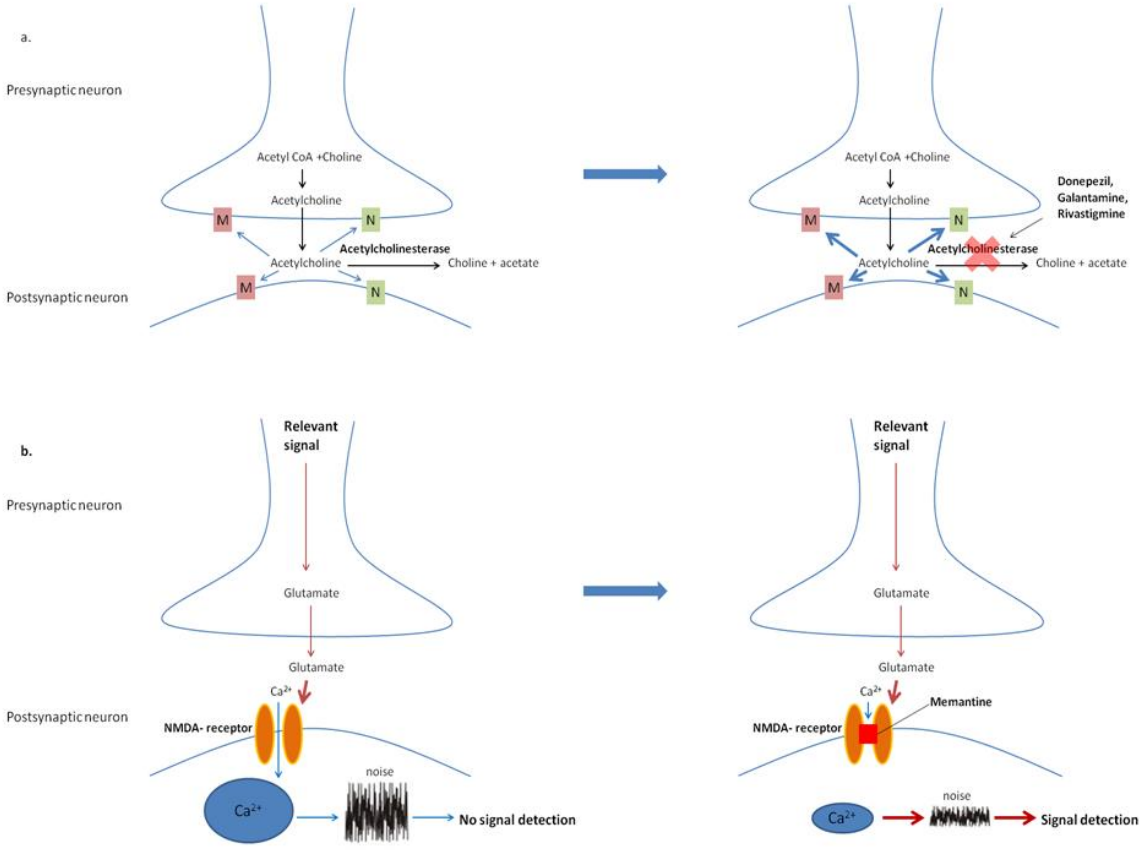

Figure 2: Mode of action of AD drugs. **a.** Acetylcholinesterase inhibitors are blocking the enzyme actylcholinesterase, responsible for the degradation of the neurotransmitter acetylcholine, thereby increasing the concentrations of acetylcholine in the synaptic cleft. N=nicotinic and M=muscarinic acetylcholine receptors. **b.** In AD, NMDA receptors channels are excessively stimulated by glutamate, which lead to constantly high intracellular calcium (Ca^{2+}) levels. In turn, synaptic noise rises, impairing relevant signal detection such as learning. Memantine is a low affinity receptor antagonist blocking open NMDA receptor channels. The intracellular Ca^{2+} level and, therefore, the synaptic noise are decreased, which allows incoming signals to be detected. Pictures adapted from Wilkinson DG, Drugs Aging, 2004;21(7):453-78 and Parsons CG, Neuropharmacology, 2004;430(7000):631-9.

Acetylcholinesterase inhibitors and memantine offer primarily symptomatic benefits regarding cognition and global change, and they might slow down, but do not prevent, the progression of the disease.[6,14,15] Numerous disease-modifying approaches are under investigations. The majority of them are targeting the production and clearance of Aβ and the abnormal aggregation of tau filaments, but also neuro-inflammatory pathways and oxidative stress are considered (Figure 3).[6] Large multicentre trials conducted to study the efficacy of disease-modifying therapies did not provide the beneficial effects hoped for.[14,16] It is expected that an early diagnosis of the disease, when no irreversible cognitive deficits are present, would lead to more positive results.[14,16] However, since AD is a complex multifactorial disorder, the discovery of a sole cure is unlikely. A treatment strategy based on multiple targets, eventually combined with a symptomatic therapy, is more probable.[6,14] Therefore, continued search for causative factors and new therapies, as well as for biomarkers for an early diagnosis, is required.

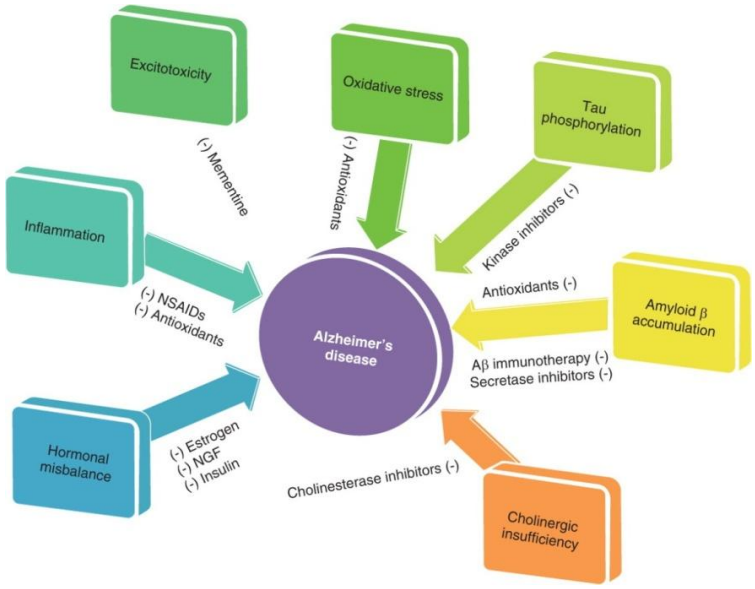

Figure 3: Current pharmacotherapeutic targets in Alzheimer's disease. Picture from Chopra K, Expert Opin Pharmacother. 2011;12(3):335-50.

1.2 General aspects of clinical pharmacology

1.2.1 Pharmacokinetics and pharmacodynamics

The objective of a pharmacological treatment is to administer effective drugs to patients while minimizing the occurrence of side effects. After drug application, there is an interplay between two overlaying processes: the pharmacokinetics, which refers to the time-course of the drug in the body, including absorption, distribution, metabolism and elimination, and the pharmacodynamics, which refers to the relationship between concentration and intensity of the therapeutic and adverse effects

(Figure 4).[17] The basis of clinical pharmacology is the fact that the intensity of many pharmacological effects depends on the amount of drug in the body and more specifically on the drug's concentration at the site of action.[18] For numerous directly and reversibly acting drugs, it has been shown that the intensity and time course of the effect is correlated with the time course of the plasma concentrations.[18] This direct relationship allows the prediction of the pharmacologic effects based on a patient's drug plasma concentration measurement. However, more complex correlations between the plasma concentration, concentration at the site of action and drug effects are possible.[18] Examples are a time delay between the attainment of effective plasma concentrations and drug action due to a retarded distribution of the compound to the site of action or time consuming synthesis or degradation of an endogenous substance necessary for the drug effect.[18] Moreover, for some drugs time-dependent change in effect intensities without changes in drug concentrations occurs through phenomena known as tolerance or sensitization.[18]

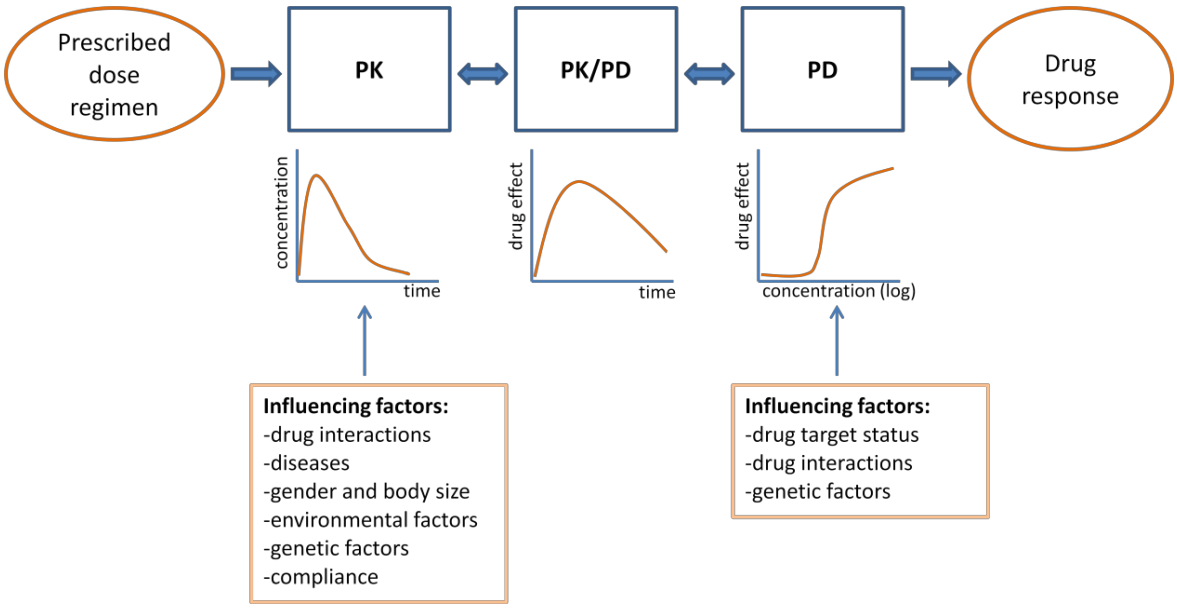

Figure 4: Relationship of pharmacokinetics and pharmacodynamics and factors affecting the two processes. PK= pharmacokinetics, PD= pharmacodynamics. Picture adapted from Derendorf H, Pharm Res, 1999;16(2):176-85.

For most drugs, substantial differences in treatment response exist among patients due to inter-individual variabilites in pharmacokinetics and pharmacodynamics. Therefore, a standard dose of a drug might not be suitable for every patient. Several factors influence the pharmacokinetics of a drug, including gender, body composition, diseases, medication, environmental factors (e.g. food or smoking) or genetic variants in metabolizing enzymes or drug transporters. Possible explanations for the pharmacodynamic variability are differences in drug receptor density or affinity, genetic factors and drug interactions (Figure 4).[19]

The development of a personalized pharmacotherapy is an approach to get closer to the aim of an optimal drug response with minimal side effects. It takes inter-individual variations in pharmacokinetics and pharmacodynamics into account in order to administer the “right drug” at the “right dose” to a particular patient.

1.2.2 Pharmacogenetics

The recognition that a part of the variability in drug response is inherited, and therefore predictable, created the field of pharmacogenetics fifty years ago.[20] With the advances in sequencing and genotyping technologies and the completion of the Human Genome Project in 2003,[21] the interest on pharmacogenetics increased sharply. The Human Genome Project was an international research effort to determine the sequence of the human genome, consisting of 3.2 billion base paires (bp), and to identify the approximately 20’000 genes that it contains.[20,21] If two unrelated individuals are compared, they differ from one another by approximately 0.1% or 3 million bp.[22] Of the DNA variants, 90-95% are single nucleotide polymorphisms (SNP), which are single nucleotide substitutions occurring at a frequency of at least 1% in the population.[20] Other DNA variants include nucleotide insertions, deletions, inversions and translocation, as well as copy number variations and variable number of tandem repeats.[23] A specific set of genetic variations, occurring together on a single chromosome or on a part of a chromosome, is called a haplotype.[22]

Currently, there are two main approaches to investigate the influence of genetic variations on drug response.[24] The candidate gene approach consists in analyzing one or more genes in which variants could plausibly explain a given phenotype.[24] Candidate genes may be in pharmacokinetic or pharmacodynamic pathways, for instance genes involved in drug metabolism, drug transport, drug targets or disease type. The second approach is based on genome-wide association studies (GWAS), in which the entire genome is screened for variations associated with a given phenotype.[24] The increasing use of the term pharmacogenomics instead of pharmacogenetics reflects the trend towards GWAS.[20] In this work, we investigated polymorphisms in candidate genes implicated in the pharmacokinetics of antidementia drugs. Moreover, we performed haplotype analyses to examine the combined effect of SNPs.

Today, the use of genotyping to support clinical decisions about drug use is not widely practiced.[25] In 2010, only about 10% of the Food and Drug Administration (FDA) approved drugs contained pharmacogenomic information in their drug labels,[26,27] thus, the potential has hardly been exhausted by now. In fact, pharmacogenetics is an emerging field with many challenges that have to be overcome, most importantly the proof of clinical utility with appropriate clinical trials, but also the cost-effectiveness, ethical issues regarding genetic analyses and the acceptance of genotyping in clinical practice.[24,25]

1.2.2.1 Drug Metabolism

The elimination of most drugs involves the participation of several families of drug metabolizing enzymes that convert hydrophobic drugs to more polar forms that are more readily excreted. The biotransformation can be divided in phase I and phase II metabolism. In phase I metabolic reactions a functional group is introduced into the parent molecule and in phase II metabolic reactions the parent molecule and phase I metabolites are further converted by conjugation with hydrophilic endogenous moieties.[28] Phase I metabolism is primarily mediated by cytochrome P450 (CYP) enzymes, whereas phase II metabolism involves a larger number of enzyme families, including the UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), *N*-acetyltransferases (NATs) and glutathione *S*-transferases (GSTs).[28]

Around 90% of drugs are metabolized by enzymes of the CYP families 1 to 3.[28] The genes of these enzymes are highly polymorphic and alleles leading to decreased or increased enzyme activity as well as to loss of function have been described. On the "Home Page of the Human Cytochrome P450 Allele Nomenclature Committee" known CYP alleles are listed and their impact on enzyme activity is indicated.[29] In addition, genetic polymorphisms have been reported, that affect the activity of the cytochrome P450 oxidoreductase (POR).[30] POR is a protein that transfers electrons from NADPH to the CYP enzymes and might, therefore, be a general limiting factor for drug metabolizing capacity.[31] Genetic variations influencing the activity of phase II enzymes have been described. It appears that they have less impact on drug clearance because phase I enzymes usually mediate the rate-limiting steps in overall drug biotransformation.[28] However, it should be mentioned that they have been less studied than CYP enzymes.[28] The importance of genetic variations in drug-metabolizing enzymes is reflected in the above-mentioned FDA list of pharmacogenomic biomarkers included in drug labels.[27] The majority of them concern enzymes of phase I or phase II metabolism.[27]

While for some enzymes genotyping is a valuable tool to predict the phenotype (e.g. CYP2D6), for others the large inter-individual variability in enzyme activity could only be partially explained by genetic variations (e.g. CYP3A4) and phenotyping by probe drugs may be more appropriate.[32-34] Phenotyping describes the metabolic situation of the patient at the moment of the test and may be influenced by environmental factors such as comedications or smoking. Depending on the clinical question, this can be an advantage. In contrast, the results of genotyping have a lifetime value as they are not affected by environmental factors, and it can be carried out in any situation.[32]

The acetylcholinesterase inhibitors donepezil and galantamine are mainly metabolized by the CYP2D6 and CYP3A, whereas rivastigmine is metabolized by its target enzymes acetylcholinesterase and butyrylcholinesterase.[11] Variations in the genes encoding CYP2D6, CYP3A and the cholinesterases have been investigated in some pharmacogenetic studies on these drugs. The current findings of these investigations are summarized in Article I. Memantine, in contrast, is only metabolized to a minor extent; it is mainly excreted unchanged by the kidneys.[35]

1.2.2.2 Drug transporter

Many drugs are transported across biological membranes via passive diffusion at a rate related to their lipophilicity.[36] However, a growing number of membrane transporters involved in the cellular uptake or efflux of a large variety of drugs has been characterized in almost all tissues, and their important role in drug delivery and disposition has been recognized (Figure 5).[37] Likewise drug-metabolizing enzymes, drug transporters are implicated in the detoxification systems of the body and may work in synergy. Uptake transporters help delivering the drugs to the metabolizing system, whereas efflux transporters help with excreting the drugs from the body and thereby decrease the load on detoxification enzymes.[37] Moreover, the chemical modification of the drugs by the enzymes increases their hydrophilicity and provides the transporters with better substrates.[37] There are two major transporter superfamilies, the solute carrier (SLC) and the ATP-binding cassette (ABC) families. SLC transporters allow the passage of drugs across the membrane by passive transport down their electrochemical gradients or by ion coupled symporters or antiporters, and the ABC transporters actively pump drugs out of the cells by using the energy of ATP hydrolysis.[38]

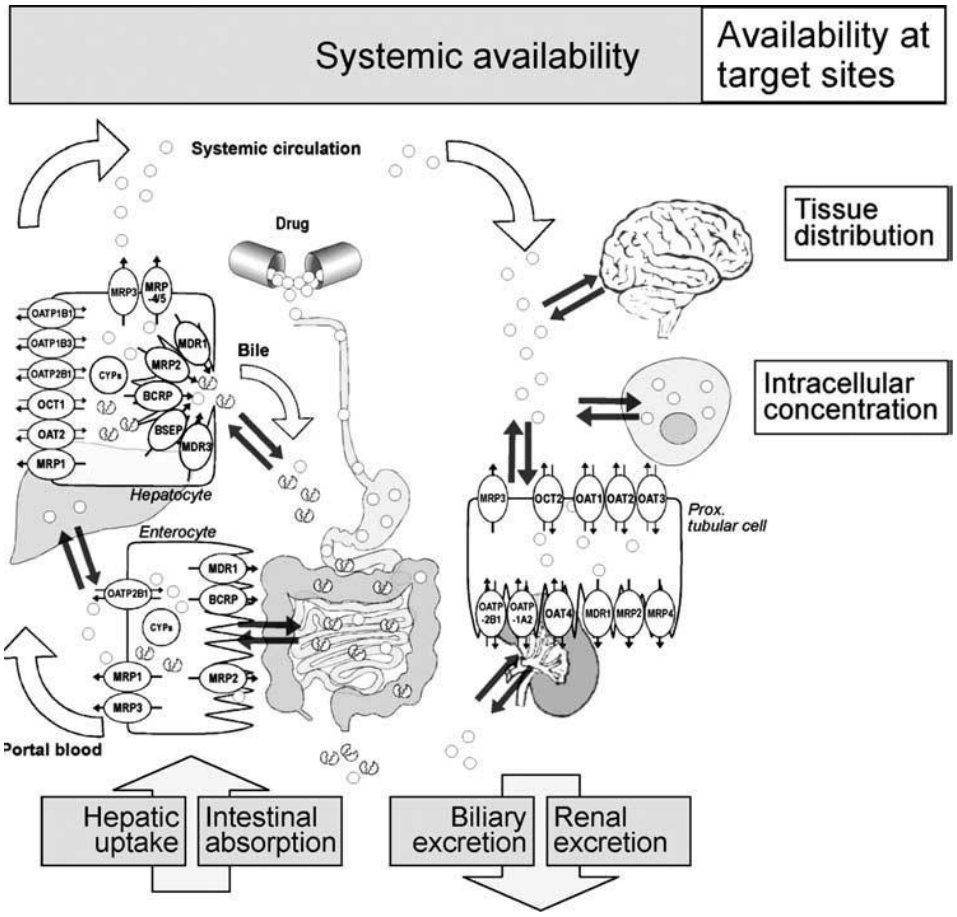

Figure 5: Schematic representation of drug uptake and efflux transporters as determinants of drug disposition. Picture from Kerb R, Cancer Lett. 2006;234(1):4-33.

The field of pharmacogenetics began with a focus on drug metabolizing enzymes, but it has been extended to membrane transporters that influence drug absorption, distribution and excretion.[37] Up to now, convincing evidence of functional relevance for most of the transporter variants is still emerging or equivocal.[39] Several reasons are conceivable for the current lack of conclusive findings. First of all, the most common genetic variants in transporters do not result in severe consequences such as a complete loss of function. Therefore, the resulting phenotypes are more subtle than those observed for inactivating mutations in metabolizing enzymes.[39] Furthermore, transporters show extensive overlap in their substrate specificity. It is therefore likely that other transporters can compensate for single failure.[37] Finally, transporter expression shows strong inter-individual differences, probably due to the regulation by several upstream signal transduction pathways and to the influence of non-genetic factors, such as sex hormones.[39] Taking this into account, a more integrative approach might be required, considering the interaction of uptake and efflux transporters, metabolizing enzymes and pathways involved in gene expression.[37,39]

Donepezil and galantamine are substrates of the P-glycoprotein (P-gp) transporter,[40,41] belonging to the ABC family. P-gp is located in tissues with excretory function, including intestine, liver and kidney, and the blood-brain-barrier.[42] Memantine is a substrate of the organic cation transporter 2 (OCT2), which belongs to the SLC family.[43] OCT2 is predominantly expressed in the kidney[36] and might, therefore, play an important role in the renal elimination of memantine. For both transporters, genetic variants have been described that alter protein expression and/or function. More details on the current pharmacogenetic research on drug transporters with respect to antidementia drugs are reported in Article I.

1.2.2.3 Nuclear Receptors

It is recognized that nuclear receptors, such as the Pregnane-X-Receptor (PXR) or the constitutive androstane receptor (CAR), are involved in the gene expression regulation of metabolizing enzymes and transporters (Figure 6).[44] Moreover, there is emerging evidence that polymorphisms in nuclear receptors influence the expression of CYPs, as it has been shown for CYP3A4 and CYP1A2.[45-47] For example, *in vitro* studies revealed genetic variants in the regulatory region of *NR1I2,* encoding the nuclear receptor PXR, which are associated with inducible and constitutive CYP3A4 expression.[45,47] Furthermore, *in vivo* studies demonstrated the influence of genetic variants in *NR1I2* on the pharmacokinetics of drugs that are CYP3A substrates.[48-50] A similar effect of genetic variations in nuclear receptors on drug transporter expression might be hypothesized, potentially influencing drug disposition.

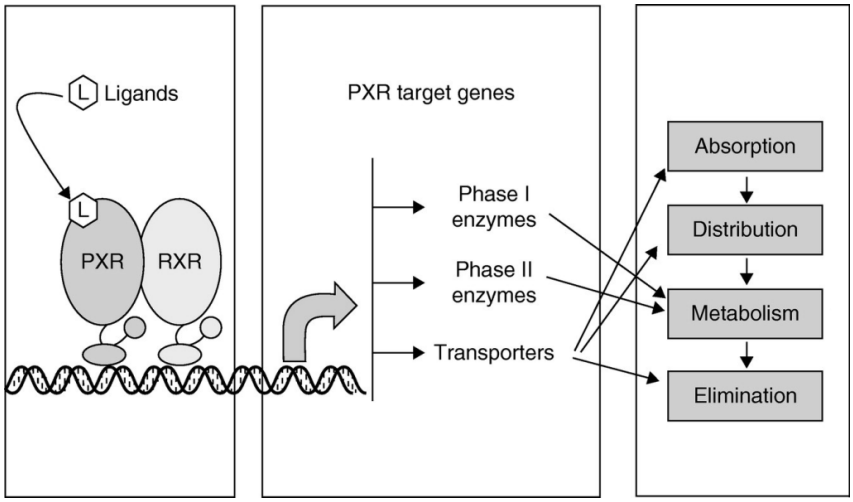

Figure 6: Schematic representation of the gene expression regulation by nuclear receptors, PXR serves as example. Following ligand binding, PXR forms a heterodimer with the Retinoid-X-Receptor (RXR) and subsequently binds to the PXR response elements in the target genes (Phase I and Phase II enzymes, transporters), inducing their expression. Picture adapted from Ma X, Expert Opin Drug Metab Toxicol. 2008 Jul;4(7):895-908.

To date, no pharmacogenetic studies have been published that investigated the influence of polymorphisms in genes of nuclear receptors on the pharmacokinetics of antidementia drugs.

1.2.3 Dose optimization trough Therapeutic Drug Monitoring

The determination of the optimal dosage for a given treatment is generally performed by relating the therapeutic response to the administered dose. However, the blood concentration of a drug can be a better predictor of the pharmacologic effect(s) than the dose, as it takes inter-individual differences in pharmacokinetic processes into account. Therefore, Therapeutic Drug Monitoring (TDM), the measurement and interpretation of drug concentrations, has found wide acceptance.[51] It has been shown that by maintaining patients drug plasma concentrations in the target range through individual dose adaption, efficacy and safety of many treatments, including psychotropic drugs, can be improved.[32,51]

Presently, no TDM analyses are performed for antidementia drugs in clinical routine. Even though little evidence exists, several factors indicate that TDM might be beneficial and in recent consensus guidelines, therapeutic ranges for these drugs have been proposed.[32] A high inter-individual variability in response to treatment has been observed,[52-54] which might partly be due to high inter-individual variabilites in plasma concentrations.[55-57] In elderly people, the presence of comorbidities and multiple comedication leading to drug-drug interactions, as well as genetic variations in metabolizing enzymes and transporters, might be causes of the observed inter-individual variabilities in plasma

concentrations. Moreover, non-adherence to the treatment could be revealed by TDM, which is a particular problem in patients with cognitive deficits.[58]

The clinical efficacy of TDM is strongly associated with appropriate pharmacokinetic interpretation of the drug measurement and is reflected by individualized dose recommendations.[51] For this purpose, the understanding of the dose-concentration relationship is essential. Population pharmacokinetic analyses are an important tool in TDM. Using Bayesian modeling, the extent of variability in pharmacokinetic parameters in a population sample is investigated and factors influencing this variability are identified.[59] In contrast to traditional pharmacokinetic studies, including healthy volunteers or highly selected patients, population pharmacokinetic analyses collect relevant pharmacokinetic information in patients who are representative of the target population and it recognize variability as important feature that should be quantified.[59] Moreover, data from sparse blood sampling can be used. This enables pharmacokinetic investigations in special populations, such as elderly patients, where the number of samples to be obtained per subject is limited because of ethical and/or medical concerns.[59] The population pharmacokinetic parameters provide a framework for optimum dosing strategies in a population, and the identified influencing factors allow the elaboration of dose recommendations in subgroup of patients.[59] Moreover, the extent of unexplained variability is determined, which is important because the efficacy and safety of a drug may decrease as unexplainable variability increases.[59] Finally, with an established population model, individual pharmacokinetic parameters of a patient can be estimated based on a random plasma concentration measurement. This information can further be used for dosage individualization in TDM.[59]

1.3 Article I: Pharmacodynamic, pharmacokinetic and pharmacogenetic aspects of drugs used in the treatment of Alzheimer's disease

Summary

The aim of this review is to summarize the pharmacodynamics and pharmacokinetics of the four commonly used antidementia drugs and to give an overview on the current knowledge of pharmacogenetics in this field.

Donepezil, galantamine and rivastigmine are acetylcholinesterase inhibitors with different pharmacodynamic and pharmacokinetic profiles. Donepezil inhibits selectively the acetylcholinesterase and has a long half-life of 70 hours. Galantamine is also a selective acetylcholinesterase inhibitor, but modulates as well presynaptic nicotinic receptors. It has a half-life of 6 to 8 h. Donepezil and galantamine are mainly metabolized by CYP2D6 and CYP3A4 in the liver. Rivastigmine is a so called "pseudo-irreversible" inhibitor of the actylcholinesterase and the butyrylcholinesterase. The plasma half-life of the drug is very short (1 to 2 h), but the duration of action is longer as the enzymes are blocked for around 8.5 h and 3.5 h, respectively. Rivastigmine is metabolized by esterases in liver and intestine. Memantine is a non–competitive low affinity antagonist of the NMDA receptor with a half-life of 70 h. Its major route of elimination is unchanged *via* the kidneys.

Addressing the issue of inter-patient variability in treatment response might be of special importance for the vulnerable population taking antidementia drugs. Pharmacogenetic considerations might help to avoid multiple medication changes due to non-response and/or adverse events. Some pharmacogenetic studies conducted on donepezil and galantamine reported an influence of the CYP2D6 genotype on the pharmacokinetics of the drugs and/or on the response to treatment. Moreover, polymorphisms in genes of the cholinergic markers acetylcholinesterase, butyrylcholinesterase, choline acetyltransferase and paraxonase were found to be associated with better clinical response to acetylcholinesterase inhibitors. However, confirmation studies in larger collectives are necessary to establish evidence of which subgroups of patients will most likely benefit from antidementia drugs.

Review article in preparation

Review Article

PHARMACODYNAMIC, PHARMACOKINETIC AND PHARMACOGENETIC ASPECTS OF DRUGS USED IN THE TREATMENT OF ALZHEIMER'S DISEASE

Muriel Noetzli[1], Chin B Eap[1,2]

1. Unit of Pharmacogenetics and Clinical Psychopharmacology, Centre for Psychiatric Neuroscience, Department of Psychiatry, CHUV, University of Lausanne, Hospital of Cery, Prilly, Switzerland.
2. Department of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Boulevard d'Yvoy 20, 1211 Geneva 4, Switzerland.

Author contributions

Concept: C.B. Eap

Literature Research: M. Noetzli

Drafting of the manuscript: M. Noetzli

Critical revision of the manuscript for important intellectual content: C.B. Eap

CONTENTS

Abstract

With the aging population, Alzheimer's disease has become a major public concern in developed countries. To date, the pharmacological treatment is symptomatic and based on the observed neurotransmitter disturbances. The four mainly used drugs are the acetylcholinesterase inhibitors donepezil, galantamine and rivastigmine, and the NMDA receptor antagonist memantine. Pharmacogenetic studies have been conducted to evaluate the influence of genetic predisposition on the inter-patient variability in response to antidementia drugs. Since comorbidities, drug interactions and non-compliance are substantial therapeutic problems in the aging population, pharmacogenetic considerations could be of special importance to avoid multiple medication changes due to non-response and/or adverse events. In this review we give an overview on the pharmacodynamic and pharmacokinetic profiles of antidementia drugs and we summarize the current knowledge of pharmacogenetics in this field.

Introduction

Dementia is a syndrome of acquired cognitive deficits, characterized by various central neurodegenerative and ischemic processes, sufficient to interfere with social and occupational functioning.[1] With the rapid global increase of the aging population, dementia has become an important public health challenge. It is estimated that currently around 24 million people suffer from dementia worldwide.[2,3] The prevalence of dementia is age-specific and almost doubles every 5 years, from approximately 1.5% in individuals aged 60-69 years to 40% in people aged over 90 years.[2] Alzheimer's disease (AD) is the most common type of dementia, accounting for 50-60% of all cases, followed by vascular dementia, Lewy body dementia and mixed dementia (coexistence of AD and vascular dementia).[1,4]

AD is a complex multifactorial and polygenetic disease. The key features of AD are formations of amyloid plaques by oligomerisation of beta-amyloid (Aβ) proteins and neurofibrillary tangles by aggregation of tau protein in the medial temporal lobe structures and cortical areas of the brain.[4] These neuropathological lesions are leading to a degeneration of neurons and synapses. It is estimated that this process already starts 20-30 years before onset of clinical symptoms.[4] Increasing evidence exists that Aβ proteins interfere with certain neurotransmitter systems, including the cholinergic and glutamatergic neurotransmission, known to have important roles in learning and memory. These findings provide a pathophysiological basis of the observed neurochemical deficits in AD.[5] Over 20 years ago, the "cholinergic hypothesis" was formulated, that states that a loss of cholinergic function in the central nervous system (CNS) contributes significantly to the cognitive decline associated with advanced age and AD.[6] The cholinergic hypofunction is linked to a reduction in a number of cholinergic markers such as the choline acetyltransferase (ChAT), muscarinic and nicotinic acetylcholine receptor binding as well as levels of acetylcholine (ACh) in the synaptic clefts.[7] In addition to the cholinergic hypofunction, the glutamate metabolism has an important impact on the cognitive functions of the brain.[4,8] There is good evidence that glutamatergic overstimulation of the postsynaptic N-methyl-D-aspartate (NMDA) receptors is implicated in the pathogenesis of AD and may results in neuronal damage.[8-10]

Up to date, mutations in the *APP* gene coding for the amyloid precursor protein (APP) and in the presenilin genes (*PSEN1* and *PSEN2*), coding for proteins involved amongst others in APP cleavage,[11] are known to be associated with the early-onset familial form of AD.[12] In contrast, for the late-onset sporadic form of AD, only the APOε4 allele of the apolipoprotein E gene (*APOE*), that codes for a protein with different functions including lipid homeostasis and Aβ scavenging, has been recognized as major risk factor for the disease.[4,12] However, with the advances in high-throughput genomic association studies, novel risk factors have been identified (e.g. gene variants in the clusterin gene (*CLU*), complement receptor type 1 gene (*CR1*), phosphatidylinositol-binding clathrin assembly protein gene (*PICALM*)), for review see [3,13,14]).

To date, the treatment of AD is based on the above mentioned disturbances of the neurotransmitters ACh and glutamate. Three acetylcholinesterase inhibitors (AChEIs) are currently used, namely donepezil, galantamine and rivastigmine, which are licensed for the treatment of mild to moderate AD.

Donepezil has as well the indication for treatment of severe stages of the disease in the US.[15] Additionally, rivastigmine is approved for the use in mild to moderate dementia associated with Parkinson's disease by regulatory agencies such as the Food and Drug Administration (FDA), the European Medicines Agency (EMEA) and SwissMedic.[15-17] Tacrine, the first AChEI on market, is associated with serious side effects, including hepatotoxicity, and has largely been replaced with the other available AChEIs.[18] The NMDA receptor antagonist memantine is licensed for moderate to severe AD and acts on the glutamate transmission.

Meta-analyses report consistent benefits of antidementia drugs regarding cognition and global assessment in AD, but with small treatment effects.[19-21] Depending on measurement scales and duration of study period, it has been shown that the proportions of responders to AChEIs and memantine were only 15-35% and around 30%, respectively.[10,22-25] Furthermore, AChEIs have non negligible adverse effects that occur at a rate of 8% in excess to placebo, the most frequent are nausea, vomiting and diarrhea.[21,24] Memantine is generally well tolerated with dizziness, headache, constipation, somnolence and hypertension as side effects with slightly higher incidence than placebo.[26]

The person-to-person variability of a drug response is a major problem in clinical practice leading to therapeutic failure or adverse effects of drugs in individuals or subpopulation of patients.[27] The source of this variability is likely to be manifold, but genetic variations in drug-metabolizing enzymes, drug transporters and drug receptors can have a significant impact. Comorbidities, drug-drug interactions, adverse reactions, and non compliance are important problems in the pharmacological management of elderly patients. [28] It would therefore be particularly useful for this population to avoid multiple medication changes due to non-response or side effects.[28] Pharmacogenetic considerations for antidementia drugs could therefore be of special interest as its understanding might be beneficial for prediction of drug efficacy and toxicity in the individual patient. Even though some pharmacogenetic studies have been conducted on antidementia drugs, by now there is no strong evidence of which subgroups of patients have favorable response to treatment or are at higher risks to develop side effects.

The aim of this review is to summarize the pharmacodynamics and pharmacokinetics of antidementia drugs and to give an overview on the current knowledge of pharmacogenetics in this field.

1. Pharmacodynamics

In Table 1 are summarized the pharmacodynamic properties of antidementia drugs.

1.1 Acetylcholinesterase Inhibitors

The main pharmacological action of donepezil, galantamine and rivastigmine is to decrease the breakdown of acetylcholine in the synapse through inhibition of cholinesterases. In humans, two major forms of cholinesterases (ChE) are found, AChE and butyrylcholinesterase (BuChE).[29] In the CNS, the

AChE activity is higher than the BuChE activity, whereas in the periphery the BuChE is the predominant form.[29,30] The principal function of the AChE is to terminate the action of acetylcholine by hydrolyzing it to acetate and choline.[31] The brain AChE is present in two globular forms, the tetrameric G4 form and the monomeric G1 form.[31] Whereas AChE is highly selective for ACh, BuChE is also able to metabolize various other molecules.[32] The physiological function of the BuChE is not clear, but it is conceivable that it acts as a scavenger, detoxifying certain chemicals and thereby limiting their entry to the CNS.[31] In the course of AD, a selective reduction of the AChE G4 form from the mild to the severe stage is seen, while the activity of the G1 form is relatively preserved.[31,32] In contrast, the activity of BuChE remains unchanged or is even increased.[30]

The three currently used cholinesterase inhibitors (ChEI) differ in their pharmacological properties. Donepezil is a non-competitive and rapidly reversible inhibitor with a 300-fold higher selectivity for AChE than for BuChE.[33,34] Galantamine is as well a reversible inhibitor of AChE, but binds to the enzyme in a competitive manner.[35] Its selectivity for AChE is 50-fold higher than for BuChE.[36] In addition to the effect on the AChE, galantamine modulates presynaptic nicotinic receptors, thereby further enhancing cholinergic activity at the synapse.[37,38] Rivastigmine is described as a "pseudoirreversible" inhibitor due to its particular interaction with the ChEs. Like ACh, rivastigmine undergoes hydrolysis by the enzyme, but leaves the esteratic site of the enzyme carbamylated and therefore inactivates the central AChE and BuChE up to 8.5 and 3.5 hours, respectively.[39,40] For that reason, the short plasma half-life ($t_{1/2}$) of approximately 1-2 hours does not influence the duration of the inhibitory effect.[41]

In contrast to donepezil and galantamine, rivastigmine shows no selectivity for AChE over BuChE.[40,42] Because of a significant decrease in AChE activity in AD patients, an additional inhibition of BuChE might be beneficial.[29,31] Another particularity of rivastigmine is its preferential inhibition of the AChE G1 form.[31,40] Differences between the three AChEIs are also seen in the central versus peripheral selectivity. In contrast to galantamine, donepezil and rivastigmine demonstrated a preferential inhibition of the central AChE.[37,39,43] The different selectivities of the drugs, AChE over BuChE, G1 over G4 form and central over peripheral, are thought to affect their efficacy and side effect profile.[32] However, meta-analyses do not reveal significant differences with respect to efficacy of the three AChEIs, but donepezil is associated with fewer side effects compared to rivastigmine and galantamine.[20,21,24] Finally, recent *in vitro* and *in vivo* data show, that ChEIs not only increase ACh levels in the synapses but could as well modulate APP processing. The resultant reduction of Aβ deposition might be important for delaying the progressive cognitive decline.[44-47]

1.2 Memantine

As NMDA receptor antagonist, memantine acts on the glutamatergic system. Glutamate is the major excitatory neurotransmitter binding to a family of ionotropic receptors, among others the NMDA receptor.[48] It is recognized that blockade of NMDA receptors leads to impairment of learning processes while their over-activation leads as well to disturbances in learning and, in a further step, to neurotoxicity.[48] Therefore, the homeostasis in the glutamatergic system is very important.[48] Excessive

activation of NMDA receptors is thought to be involved in AD.[5,48,49] It is supposed that the continuous activity of the receptors results in a raise of synaptic noise, impairing detection of relevant signals such as learning.[48] In AD, magnesium, an endogenous inhibitor which normally works as filter to detect relevant signals, is too weak to serve this role.[50]

Before introduction of memantine, clinical trials with NMDA receptors antagonists have failed due to high binding affinity towards NMDA receptors or unspecificity of the drugs leading to unacceptable side effects.[49] Memantine has a more favorable side-effect profile because of its special features as non-competitive low affinity antagonist. Since it binds only to open channels, a pathological increased NMDA receptor activity, with resultant excessive channel openings, is inhibited to a greater extent than physiological receptor activity.[51] Moreover, the low binding affinity ensures that the drug does not accumulate in the ion channels, and therefore, does not block subsequent synaptic neurotransmission.[51] With these characteristics, memantine is thought to act as a slightly more effective surrogate for the intrinsic NMDA inhibitor magnesium.[48] Clinical trials on memantine are focused on improvements in cognitive function and behavioral aspects, but memantine is potentially as well a neuroprotective agent.[50,51]

Given the complementary mode of action, different studies were conducted to investigate the effect of concomitant administration of memantine with AChEIs. The majority of these studies revealed a beneficial effect of coadministration of the two drug classes on different measurement scales, also on a long-term basis.[52-56]

2. Pharmacokinetics

In Table 2 are shown the steady-state pharmacokinetic parameters of antidementia drugs.

2.1 Donepezil

2.1.1 Pharmacokinetic parameters and metabolism

Donepezil is well absorbed with a bioavailability approaching 100% and peak plasma concentrations (C_{max}) are achieved after 3-5 h.[57,58] Food intake has no significant effect on the drug absorption.[58] The drug demonstrates linear kinetic properties with a $t_{½}$ of approximately 70 h indicating that once daily administration is appropriate and that steady-state conditions are reached within two to three weeks.[59] The mean serum protein binding of the compound is around 93% and the volume of distribution (V) is approximately 12 L/kg. [58,60,61]

Donepezil is metabolized hepatically by the enzymes cytochrome P450 (CYP) 3A4 and 2D6 and the primary route of elimination of the parent drug and the metabolites is renal.[62] There are three suggested metabolic pathways: *O*-demethylation to the metabolites M1 and M2 followed by glucuronide conjugation to the metabolites M11 and M12, *N*-dealkylation to the metabolite M4 and as minor route *N*-oxidation to the metabolite M6.[62,63] Unchanged donepezil was found to be the principal component in plasma and urine.[62] The metabolite M1 (6-*O*-desmethyl-donepezil) is the only active

metabolite with a comparable potency in AChE inhibition as donepezil. In human plasma, M1 is only present at about 20% of the concentration of the parent drug.[57] Moreover, in rats the transfer of M1 into the brain as target organ is very low.[64] Therefore, it is suggested that M1 does not significantly contribute to the pharmacological activity of the drug.

Due to its CYP dependent metabolism, it is suggested that drug interactions via CYP3A4 and CYP2D6 could be important. Two studies in healthy volunteers showed an influence of CYP inhibitors on donepezil plasma levels. The concurrent administration of ketoconazole, a strong CYP3A4 inhibitor, resulted in a significant increase in donepezil plasma concentrations, estimated to be 23-30% at steady state.[65] Moreover, the coadministration of cimetidine, a CYP3A4 and CYP2D6 inhibitor, leaded to slightly higher C_{max} and area under the curve (AUC) values (11-13% and 10%, respectively).[66] In both studies the $t_{1/2}$ and time to maximum peak plasma concentration (t_{max}) of donepezil were not significantly altered.[65,66] The clinical significance of these interactions remains to be determined, but careful monitoring is recommended when donepezil is coadministrated with CYP3A4 and CYP2D6 inhibitors.

2.1.2 Effects of age, renal and hepatic impairment on the pharmacokinetics

Results of a single dose study in healthy young volunteers (age 20-27 years) and elderly patients (age 65-82 years) suggest that some of the pharmacokinetic parameters are affected by aging due to a slower absorption and a wider distribution of the drug.[61] The $t_{1/2}$, t_{max} and V in steady state conditions are significantly higher in the elderly than in the young. However, as the AUC, C_{max} and the clearance (CL) did not differ between the two groups, modification of the dose in elderly does not appear to be necessary.[61]

As donepezil and its metabolites are predominantly renally cleared, a rise in plasma concentrations could be expected in subject with impaired kidney function.[67] Nevertheless, in a single and a multiple dose study with 5 mg donepezil daily, no significant differences in pharmacokinetic parameters were found between healthy controls and subjects with moderately to severely (CL_{creat} 7-30 mL/min/1.73m^2) and moderately (CL_{creat} 17-33 mL/min/1.73m^2) impaired renal function, respectively.[67,68] Therefore, no dose adjustment is required for patients with moderate renal insufficiency. In contrast, at steady state mean values for AUC, $t_{1/2}$, C_{max} and the mean concentration at steady-state (C_{ss}) were significantly increased in subjects with impaired liver function (Child-Pugh grade A or B) compared with healthy controls.[69] It is suggested that the administration of 5 mg donepezil daily is safe and well tolerated in patients with mild to moderate hepatic impairment.[69] However, a doubling of the dose to 10 mg daily should be monitored.

2.2 Galantamine

2.2.1 Pharmacokinetic parameters and metabolism

Galantamine is well and quickly absorbed with a bioavailability approaching 100%.[70] Coadministration with food does not alter the total amount of absorbed drug substance, but t_{max} is delayed by

approximately 1.5 h and C_{max} is decreased by 25%.[71] Galantamine has a linear kinetic over a large dose range and a $t_{½}$ of around 7 h.[70,72,73] The V is 2.6 L/kg and the drug exhibits a low plasma protein binding of 17%.[70,74] An extended-release (ER) formulation of galantamine was developed allowing once-daily drug administration. The immediate-release (IR) and the extended-release (ER) formulations are bioequivalent with respect to AUC and minimum plasma concentration (C_{min}) for a daily dose of 24 mg. As expected, the ER formulation resulted in higher t_{max} values (4.4 h versus 1.2 h) and C_{max} values were reduced by 25%.[72]

Approximately 30% of galantamine is excreted unchanged in the urine; the other part is metabolized through multiple pathways, primarily by *O*-demethylation by CYP2D6, *O*-oxidation by CYP3A4 and glucuronidation.[75] After a single dose of 4 mg radiolabeled galantamine, the parent compound accounted for the bulk radioactivity in plasma.[75] Beside, two major metabolites were found, namely the glucuronide of *O*-desmethyl-galantamine and the glucuronide of galantamine itself.[75] *In vitro* it has been shown that *O*-desmethyl-galantamine is a more potent inhibitor of AChE than galantamine. However, the unconjugated metabolite was not detected in plasma and may not contribute to the pharmacological activity of the drug since it is immediately inactivated by glucuronidation.[75,76]

The effect of the strong CYP2D6 inhibitor quinidine on the metabolism of galantamine was investigated.[76] Four subjects received a single dose of 15 mg galantamine alone and 7 days later in combination with 250 mg quinidine. The inhibition of the *O*-demethylation pathway resulted in an almost complete suppression of the renal elimination of *O*-desmethyl-galantamine glucuronide. The urinary excretion of unchanged galantamine and other metabolites was consequently increased. Additionally, multiple dose pharmacokinetic studies showed an increase of the AUC by 40%, 30% and 10% when galantamine was coadministrated with paroxetine (inhibitor of CYP2D6), ketoconazole (inhibitor of CYP3A34 and CYP2D6), and erythromycin (inhibitor of CYP3A4), respectively.[77] Dose reduction may be required when galantamine is given with inhibitors of CYP2D6 and CYP3A4.

2.2.2 Effects of age, renal and hepatic impairment on the pharmacokinetics

A galantamine population pharmacokinetic study, including data from 15 clinical trials, showed a decreased galantamine CL with increasing age.[78] In AD patients the CL was, on average, reduced by 30% compared to healthy young subjects.[78] In the same study, model simulations were conducted in AD patients with renal and hepatic impairment. No significant difference in galantamine plasma concentrations were found between patients with and without renal insufficiency (CL_{creat} 9-51 mL/min).[78] In contrast, their model investigating hepatic impairment showed a 60% reduction in metabolic CL among patients with moderate hepatic dysfunction (Child-Pugh grade B) and therefore higher apparent galantamine levels.[78] These results are in line with the findings of a single dose study of 4 mg galantamine comparing healthy volunteers and subjects with mildly (Child-Pugh grade A) and moderately (Child-Pugh grade B) reduced hepatic function.[74] No significant difference in CL was found in subjects with mild hepatic dysfunction, but the CL was reduced by 23% in subjects with moderate hepatic impairment.[74] Consequently, dose titration should proceed cautiously in patients with

moderately impaired hepatic function and the use in patients with severely impaired liver function is not recommended.

2.3 Rivastigmine

2.3.1 Pharmacokinetic parameters and metabolism

After oral administration, rivastigmine is rapidly and almost completely absorbed reaching maximum plasma concentrations within 1 h.[79,80] Concomitant food slows the absorption, lowers C_{max} by 30% and increases AUC by 30% compared to fasting conditions.[39] Since it is supposed that the high peak plasma concentrations are related to gastrointestinal side effects, administration of rivastigmine with food is recommended for a better tolerability.[39] Rivastigmine exhibits non-linear pharmacokinetics due to a capacity-limited elimination.[39,79] Thus, the plasma level and AUC of the drug increase more than proportionally with increasing dose. Nonlinearity is also shown in the non proportional enhancement in absolute bioavailability of the drug. Following a single dose of 3 mg and 6 mg rivastigmine, the bioavailability is 35% and 70%, respectively.[41,81] Rivastigmine has a very short $t_{½}$ life of 1.5 to 2 h, therefore, steady state conditions are essentially reached by the second dose.[39,41,79,80] The drug is widely distributed throughout the body with a V between 1.8 and 2.7 L/kg and about 40% is bound to plasma proteins.[39,82] To improve the tolerability of rivastigmine and the patient compliance, a transdermal patch has been developed.[83,84] This formulation shows beneficial pharmacokinetic characteristics as the patch gradually releases the drug substance over the 24 h application period. Findings of a comparison study of patch for 24 h and capsules b.i.d demonstrated a lowered C_{max} by around 30% and a considerably later t_{max} (8 h versus 1 h), with a similar level of exposure.[79] Additionally, the continuous release of rivastigmine lowered the fluctuation between peak and trough plasma concentrations significantly.[79,83]
Rivastigmine is extensively metabolized by cholinesterases in the liver and to a lesser extent in the intestine to NAP 226-90, an inactive and non-toxic metabolite.[40,79,82] Renal excretion of the sulfate conjugate of NAP 226-90 is the primary route of elimination.[40] After 24 h, the drug and the metabolite are almost completely excreted.[39] Less of the metabolite NAP226-90 is formed following patch administration due to the lack of first-pass metabolism, and less inter-patient variability was observed for the treatment with the patch versus capsules.[79,83,85] Hepatic microsomal enzymes are not involved to any significant extent in the metabolism of rivastigmine, making drug-drug interactions unlikely.[80]

2.3.2 Effects of age, renal and hepatic impairment on the pharmacokinetics

Findings of a study comparing pharmacokinetics in young and elderly healthy subjects demonstrated a slightly prolonged $t_{1/2}$ in elderly persons (0.88-1.25h versus 0.80-0.99 h). However, another study in AD patients aged 50 to 92 years revealed no difference in drug absorption and exposure with age.[39] A dose escalating study with 1-3 mg rivastigmine investigated the influence of renal and hepatic impairment on the pharmacokinetics of the drug. Compared to healthy controls, the AUCs of rivastigmine and its metabolite were 1.4 fold and 1.5 fold increased in patients with severe renal

impairment (CL_{creat} <10 mL/min).[86] In patients with moderately severe liver cirrhosis, the AUCs of rivastigmine and NAP 226-90 were 2.3-fold higher and 0.8-fold lower compared to healthy controls.[39,86] However, no specific dose recommendations are given for patients with renal and hepatic impairment, because of the clinical practice of dose titration according to tolerability of the drug.[39,86]

2.4 Memantine

2.4.1 Pharmacokinetic parameters and metabolism

Memantine is well absorbed with a bioavailability close to 100%, reaching maximum plasma levels within 3-8 h.[87] The drug exhibits linear pharmacokinetics in a wide dose range with a $t_{1/2}$ between 60 and 70 hours.[88,89] Steady state conditions are attained within 11 days with plasma levels 3-4 times higher than C_{max} following a single dose.[89,90] The V is reported between 4 and 9 L/kg and the rate of plasma protein binding is approximately 45%.[87,91] Memantine and its metabolites are mainly excreted via the kidneys with contribution of tubular secretion.[87] In man, about 80% of the circulating memantine dose is present as the parent compound.[92] Main metabolites are N-3,5-dimethyl-gludantan, the isomeric mixture of 4- and 6-hydroxy-memantine, and 1-nitroso-3,5-dimethyl-adamantane, none of which is active.[92] Despite the fact that memantine undergoes hydroxylation and oxidation, typical CYP-catalyzed reactions, no contribution of these enzymes have yet been detected.[93] However, it has been found that memantine is a potent and selective inhibitor of the CYP2B6 enzyme at clinically relevant concentration.[93] Tubular secretion of memantine occurs via the renal cation transport system.[87] Memantine has been shown to be a substrate of the organic cation transporter 2 (OCT2),[94] which is predominantly expressed in human kidneys.[95] Therefore, OCT2 might play an important role in memantine elimination. Urine pH has been shown to be a major determinant for the renal excretion of alkaline drugs like memantine.[96] A clinical trial with healthy volunteers demonstrated a 7-10 fold higher renal CL in alkaline urine (pH 8) compared with acidic urine (pH 5).[96] To alkalize the urine the subjects received sodium bicarbonate, and for acidification ammonium chloride was administered. Nevertheless, in a more naturalistic approach measuring the urine pH of a representative patient's population, no significant effect of urine pH (pH range 4.9-9) on memantine CL was found.[91]

Co-medication with drugs which are eliminated via tubular secretion resulted in a lower CL of memantine.[91] A competitive drug-memantine interaction at the OCT2 in the kidney could be a possible explanation, even though the memantine concentrations necessary for the interaction with OCT2 are higher than the therapeutic serum concentrations. However, it is conceivable that other renal transporters are implicated in the observed drug interactions.[91] Another study investigated the pharmacokinetic interaction of memantine and glyburide/metformin.[97] Healthy subjects received a single dose of memantine with and without previous administration of glyburide/metformin for 6 days. Although metformin is cleared renally via organic cation transporters, no pharmacokinetic interaction between metformin and memantine was found.[97]

2.4.2 Effects of age, renal and hepatic impairment on the pharmacokinetics

Considering studies conducted in young and elderly healthy subjects, the pharmacokinetic parameters changed only slightly with age, most probably due to variations in body weight and fat.[87]
Significant differences were seen in patients with moderate (CL_{creat} 30-49 mL/min) to severe (CL_{creat} 5-29 mL/min) renal impairment compared to healthy subjects regarding AUC (increased by 60% and 115%) and CL (decreased by 36% and 52%). In addition, $t_{1/2}$ was enhanced by 95% in subjects with severe renal impairment.[88] According to the prescription information, memantine dose can be increased up to the normal dose of 20 mg per day if 10 mg is well tolerated during the titration phase.[92] In patients with severe renal impairment, half of the normal daily memantine dose is recommended. As memantine is metabolized only to a minor extent and into inactive compounds, no clinically relevant changes in pharmacokinetics are expected in patients with hepatic impairment.[87]

3. Pharmacogenetics

3.1 Acetylcholinesterase Inhibitors

It is widely accepted that genetic variations in drug metabolizing enzymes contribute to therapeutic failures and adverse drug reactions. With regard to the metabolism of donepezil and galantamine, the liver enzymes CYP2D6 and CYP3A4 are interesting candidates for pharmacogenetic investigations.[18] Genetic polymorphisms in *CYP2D6* are extensively studied in many different populations and over 80 allelic variants have been described.[98] Phenotypically four types of metabolizers can be distinguished with different frequencies in the Caucasian population: poor metabolizers (PM), intermediate metabolizers (IM), extensive metabolizers (EM) and ultrarapid metabolizers (UM) accounting for approximately 5-10%, 10-17%, 70-80% and 3-5%, respectively.[99,100] The PM phenotype is characterized by the presence of two null alleles, which do not encode a functional protein. Consequently, they are unable to use the CYP2D6-dependent metabolic pathway for drug elimination which can lead to very high plasma concentrations and even toxicity. The CYP-dependent metabolism is as well limited in the IM phenotype, as they usually carry a null allele in combination with a reduced-function allele. The EM phenotype is due to one or two alleles with normal function. By contrast, multiple gene copies are causing the UM phenotype.[99,100] UMs typically exhibit decreased drug plasma concentrations at conventional doses and are therefore prone to a lack of response regarding drugs metabolized by CYP2D6.
To our knowledge, only one clinical trial has been conducted investigating the impact of genetic variations in *CYP2D6* on donepezil plasma concentrations. This steady-state study in 42 patients of Caucasian ethnicity included 2 UMs, 10 heterozygous EMs (one functional *CYP2D6* allele) and 30 homozygous EMs (two functional *CYP2D6* alleles) receiving donepezil for at least 3 months.[101] An impact of the *CYP2D6* genotype on drug plasma concentrations is suggested since UMs had lower drug plasma concentrations compared to EMs, and homozygous EMs had lower plasma levels than

heterozygous EMs. However, the differences in plasma concentrations didn't reach statistical significance, possibly because only a small number of subjects and no PMs were included.[101] Interestingly, heterozygous EMs showed a significantly better clinical response to treatment than homozygous EMs, as measured by change in the MMSE score (1.4 *versus* -1.3).[101] Moreover, different clinical trials were carried out to evaluate the influence of the *CYP2D6* genotype on the response to donepezil treatment. In a prospective study including 94 Spanish AD patients, the subjects were treated with a combination therapy consisting of donepezil and three other drugs (CDP-choline, piracetam, nicergoline).[28] The best responders were the EMs and IMs with a clear improvement of cognition after one year (MMSE change 2.2 and 1.1), and the worst responders were the PMs and UMs showing a progressive cognitive decline (MMSE change -2.7 and -1.4).[28] In a prospective study including 127 Caucasian AD patients treated with donepezil, a significant higher frequency of patients with the G allele of the rs1080985 single nucleotide polymorphism (SNP) in *CYP2D6* was found in non-responders compared to responders (60% versus 35%) after 6 months.[102] Since it has been reported that the G allele is associated with a higher enzyme activity and therefore a faster metabolism, it is thought that the poorer response to donepezil is due to lower plasma levels of the drug.[102] The same group investigated 16 functional polymorphisms in *CYP2D6* on the clinical response after 6 months of treatment with donepezil in a new cohort of 57 patients with mild-to-moderate AD.[103] A significant higher frequency of gene variants conferring decreased or absent enzyme activity was observed in responders than in non-responder patients (74% versus 37%). Finally, the clinical outcome in 171 Italian AD patients treated with donepezil, galantamine or rivastigmine was investigated in a prospective study taking into account genetic variations in *CYP2D6* and *BCHE*, encoding the BuChE.[104] In this study population, no significant association was found between *CYP2D6* and *BCHE* genotypes and response to treatment after one year for all three ChEIs.[104] The discrepancy of the results regarding the impact of genetic variations in *CYP2D6* on response to donepezil treatment might be due to different follow-up periods and to different approaches to classify responders and non-responders as well as prediction of the CYP2D6 phenotypes from genotypes. However, since it is suspected that higher plasma levels could be associated with a higher response rate but possibly also with a higher frequency of side-effects, more studies are needed to clarify the influence of polymorphisms in *CYP2D6* on plasma concentrations of donepezil and the clinical response.

In three studies the impact of the *CYP2D6* genotype on galantamine pharmacokinetics was assessed. In the first study, genotyping was performed in a subset of 356 patients included in two phase III clinical trials, among 336 EMs and 20 PMs.[78] The population pharmacokinetic model indicated that the galantamine CL is reduced by 25% in PMs compared to EMs.[78] In the second study, the metabolite profile was investigated in two EMs and two PMs after administration of a single low dose of 4 mg galantamine.[75] There was a considerable difference regarding the CYP2D6 catalyzed *O*-demethylation between EMs and PMs. In EMs, six metabolites resulting from *O*-demethylation represented over 33% of the dose, whereas in PMs these metabolites accounted for only 5% of the dose. However, the lower level excretion of metabolites formed by *O*-demethylation in PMs was compensated by excretion of higher levels of unchanged galantamine and the N-oxid of galantamine. No apparent difference in

galantamine plasma levels was seen between EMs and PMs in these four subjects.[75] In another study comparing galantamine pharmacokinetic parameters in 13 Japanese with 12 Caucasian healthy volunteers, one PM was identified in the Caucasian group.[73] No apparent pharmacokinetic difference was seen in this subject. In none of these studies UMs were taken into account.

Likewise CYP2D6, the second CYP implicated in the metabolism of donepezil and galantamine, the CYP3A4, demonstrates a large inter-individual variability in expression and activity. CYP3A4 is the major form of human cytochromes in the liver and it is also abundant in the intestine.[105] To date, various genetic variations in *CYP3A4* have been described, but their predictive information on protein expression or activity is limited.[105] There is a large overlap in substrate specificity between the isoenzyme CYP3A4 and CYP3A5, therefore, it might be that donepezil and galantamine are to some extent as well metabolized by the latter. Compared to CYP3A4, CYP3A5 is expressed at a much lower level in the liver, but it has a wider tissue distribution.[105] *CYP3A5*3* is the most common allele in Caucasians with a frequency of 95% leading to a low expression of the enzyme. However, in homozygous carriers of the *CYP3A5*1* allele, the CYP3A5 can contribute up to 50% of the CYP3A content in the liver and might therefore be important in drug metabolism.[105] Furthermore, it has been shown that polymorphisms in the cytochrome P450 oxidoreductase (POR) or in the pregnane X receptor (PXR), a regulator of CYP3A transcription, could influence drug metabolism by CYP3A.[106-108]

A study in 54 Caucasian AD patients has been conducted to investigate the influence of different *CYP3A4* and *CYP3A5* alleles on the plasma levels and the clinical outcome of donepezil.[109] The results suggest that the investigated genetic variants of *CYP3A4* and *CYP3A5* do not play a pivotal role in the variability in donepezil metabolism. In contrast, an influence of polymorphisms in the *ABCB1* gene on donepezil concentrations and response to treatment was found. *ABCB1* codes for the membrane transporter P-Glycoprotein (P-gp), which has a large substrate overlap with CYP3A4 and is implicated in absorption, distribution and excretion of many drugs.[110] Patients homozygous for the *ABCB1* 1236T/2677T/3435T haplotype showed a tendency towards a better clinical response and lower plasma concentrations. However, the differences didn't reach statistical significance.[109]

Associations of genetic variations in *ACHE*, encoding the AChE, and *BCHE* with response to AChEI treatment were studied in a few clinical trials. These investigations could be especially interesting for rivastigmine since AChE and BuChE are the target enzymes and at the same time the main metabolizing enzymes for this agent. In an Italian study population of 461 AD patients, gene frequency distributions of one SNP in ACHE and two SNPs in BCHE, as well as one SNP in the gene of ChAT, were investigated.[111] Additionally, the association of these SNPs with response to either donepezil or rivastigmine treatment was assessed in a subset of 171 patients. It was found that carriers of the *ACHE* rs2571598 AA genotype receiving rivastigmine showed a slightly better response to treatment evaluated by the MMSE score changes (-1.62 for G/G, -2.71 for G/A and -1.03 for A/A). In contrast, no association was seen in patients treated with donepezil. Additionally, no influence of the K-variant of *BCHE* (rs1803274), which leads to a lower enzyme expression, was seen on the treatment effect.[111] This result is in agreement with the above mentioned study investigating genetic variations in *CYP2D*6 and *BCHE* in a cohort of 171 Italian AD patients treated for one year with donepezil, rivastigmine or galantamine.[104] However, in a retrospective analysis in 114 Caucasian AD patients younger than 75

years, a differential response to rivastigmine regarding *BCHE* genotype was found.[112] On different measurement scales wild-type *BCHE* carriers had a better response to rivastigmine treatment compared to donepezil, whereas the treatment response was similar within carriers of the K-allele.[112]
Furthermore, polymorphisms in other cholinergic markers were studied regarding responsiveness of patients to AChEI therapy. The influence of six polymorphisms in *CHAT*, encoding the enzyme responsible for ACh biosynthesis, was studied in 121 patients recruited in Northern Ireland and receiving an AChEI. The SNP rs733722, lying in a putative promoter region of the gene and appearing with a frequency of 33%, showed to be a marker of response to AChEI treatment.[113] Carriers of two T alleles had a significant smaller change in MMSE/year than subjects carrying at least one copy of the C allele (-0.18 versus -1.84).[113] Paraxonase-1 (PON-1) is an arylesterase with multiple biological functions, showing high inter-individual differences in activity.[114] It is known that the *PON1* 192Q/R polymorphism (rs662) is influencing the activity of the enzyme.[114] Interestingly, this enzyme is also a potent endogenous cholinesterase inhibitor.[114] Therefore, it might be that PON-1 interacts synergistically with drugs inhibiting ChEs.[114] A study investigated the *PON1* 192Q/R polymorphism (rs662) in 73 Caucasian patients treated with donepezil or rivastigmine.[114] The R allele was present in 44% of patients that responded to treatment, while its frequency was only 26% among non-responders, thus, suggesting that carriers of the R allele have more treatment benefits than Q allele carriers.[114] However, these results could not be confirmed by a recent study, investigation this polymorphism in 101 Polish patients.[115] Furthermore, phenotypic screening of the constitutive m1 muscarinic acetylcholine receptor activity (CHRM1) was done in a cohort of 74 individuals, including 48 diagnosed with primarily AD and stratified by their treatment response to AChEIs.[116] The authors concluded that the m1 receptor gene is not highly polymorphic in the human population suggesting that genetic variation within this gene is not a contributing factor to the clinical variability in treatment with AChEIs.[116] Further studies are needed to clarify the impact of SNPs in cholinergic markers on response to treatment with AChEIs.
In contrast, many pharmacogenetic studies were conducted to evaluate the impact of the lipoprotein *APOE* genotype on treatment response to AChEIs. There are three major polymorphic forms of APOE, ε2 (Cys112, Cys158), ε3 (Cys112, Arg158) and ε4 (Arg112, Arg158), which differ from one another by one amino acid substitution.[117] The APOε4 allele is known to be a major risk factor for the development of AD. APOE plays various roles in the CNS, it is proposed to influence the amyloid-β metabolism, lipid homoeostasis, synaptic activity, response to cellular injury, and neuroinflammation.[117] Since it is suggested that the integrity of the cholinergic system vary between carriers of the different *APOE* alleles, it was suggested that the *APOE* genotypes might be a predictor for the quality and size of drug response.[118] However, the results of the pharmacogenetic studies are conflicting and the majority of the studies report no association of the *APOE* genotype with donepezil, rivastigmine or galantamine treatment response.[119-128]

3.2 Memantine

Given the large inter-individual differences in plasma levels and the concentration dependent probability of adverse events of memantine,[87,88,91] it would be beneficial to understand the demographic and genetic factors influencing this variability. To our knowledge, no pharmacogenetic studies have been conducted on memantine. The high inter-individual variability could partly be due to differences in renal elimination as memantine is mainly excreted unchanged via the kidneys.[87] Since it is suggested that OCT2 is an important transporter involved in the active secretion of memantine, genetic variation in this transporter might cause inter-individual differences in the renal CL of the drug. *In vitro* data have revealed genetic variations in *SLC22A2,* coding for OCT2, altering protein function and expression.[129-133] The most common coding polymorphism 808G>T (rs316019), occurring at a frequency of 10 to 15% in different ethnics, has been investigated in several pharmacokinetic studies of metformin and in one clinical trial of cisplatin, both agents renally excreted by OCT2.[134-138] The results of these studies are divergent, thus, up to now no conclusion can be drawn for the predictive effect of this SNP on metformin and cisplatin pharmacokinetic parameters. However, plasma concentrations of memantine could be influenced by genetic variations in OCT2 or other renal transporters. More studies are requested to elucidate the impact of polymorphisms in drug transporters on pharmacokinetics of renally eliminated drugs.

4. Conclusion

The current treatment of AD is based on the observed neurotransmitter disturbances: the cholinergic hypofunction and the glutamatergic overstimulation of NMDA receptors. Donepezil, galantamine and rivastigmine are AChEIs, increasing the level of ACh in the synaptic clefts. The three drugs have different pharmacodynamic and pharmacokinetic profiles, therefore, efficacy and tolerability may not be identical in the individual patient and a change from one AChEI to another might be beneficial. Donepezil is a selective AChEI and has a $t_{1/2}$ of 70 h, therefore, once-daily dosing is applied. The drug is mainly metabolized by CYP2D6 and CYP3A4 in the liver. Galantamine is as well a selective AChEI but modulates as well presynaptic nicotinic receptors. It has a $t_{1/2}$ of 6 to 8 h, but once daily dosing is possible by administration of the ER formulation. Galantamine is metabolized hepatically by the CYP2D6 and CYP3A4. Rivastigmine is a so called “pseudo-irreversible” inhibitor of AChE and BuChE. The plasma $t_{1/2}$ of the drug is very short (1 to 2 h), but the duration of action is longer as the AChE and the BuChE are blocked for around 8.5 h and 3.5 h, respectively. Oral formulations of the drug have to be given twice a day, whereas the patch allows a once-daily administration. Rivastigmine is metabolized by esterases in liver and intestine. Memantine is a non–competitive low affinity antagonist of the NMDA receptor, inhibiting the pathological excessive channel openings. It has a $t_{1/2}$ of around 70 h, a once daily dosing is therefore sufficiently. Mematine is only metabolized to a minor extent, the major route of elimination is unchanged *via* the kidneys.

Pharmacogenetic studies addressing the issue of inter-patient variability in response to antidementia drugs might be valuable to develop a “personalized pharmacotherapy” with the potential to maximize the benefit of therapy and to minimize side effects. This approach could be of special importance for the population taking antidementia drugs, since it is vulnerable to comorbidities, polypharmacy and non-compliance. Some pharmacogenetic studies conducted on donepezil and galantamine reported an influence of the *CYP2D6* genotype on the pharmacokinetics of the drugs as well as on the response to treatment. Moreover, polymorphisms in genes of the cholinergic markers AChE, BuChE, ChAT and PON-1 were found to be associated with better clinical response to AChEIs. However, confirmation studies in larger collectives are necessary to establish evidence of which subgroups of patients will most likely benefit from antidementia drugs.

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Table 1: Pharmacodynamic properties of antidementia drugs.

	Cholinesterase inhibitors				**NMDA-receptor[a] antagonist**	**References**
Drug		Donepezil	Galantamine	Rivastigmine	Memantine	
Mode of action	Mode of inhibition	Non-competitive, rapidly reversible	Competitive, rapidly reversible	Non-competitive, very slowly reversible	Uncompetitive, low-affinity NMDA-receptor antagonist	32,51
	AChE/BuChE[b] selectivity	300	50	1		33,34,36,42
	Brain versus peripheral selectivity	Yes	No	Yes		31,34,40
	ACh isoform selectivity	None	None	G1>G4		31,32,40,139
	nAChR[c] modulation	No	Yes	No		32
Major side effects		Nausea, vomiting, diarrhea			Dizziness, headache, constipation, somnolence, hypertension	21,24,26

a) NMDA-receptor=N-methyl-D-aspartate-receptor
b) AChE=acetylcholinesterase, BuChE=butyrylcholinesterase
c) nAChR=nicotinic acetylcholine receptor

Table 2: Steady-state pharmacokinetic parameters of antidementia drugs.

Drug	Donepezil	Galantamine	Rivastigmine	Memantine	References
Daily dose	5-10 mg, 23 mg[a]	16-24 mg	6–12 mg	10-20 mg	
Bioavailability	100%	100%	35% (3 mg), 70 % (6 mg)	100%	41,57,70,87
Protein binding	93%	17%	40%	45%	61,74,81,87
$t_{1/2}$	70 h	6-8 h	1.5-2 h[b] (cap[c]), 3.4 h[b] (patch)	60-70	41,59,60,70,72,73,79,80,88,89
t_{maxss}	4 h	1 h (IR[c]), 4-5 h (ER[c])	1 h (cap[c]), 8 h (patch)	3-8 h	60,72,79,87,140
C_{maxss}[d] [ng ml^{-1}]	61 ± 10 (10 mg/d)	53±10 (ER[c] 16 mg/d)	30±13 (cap[c] 12 mg/d), 7.9±2.9 (patch 9.5 mg/24h)	70-180 (20 mg)	60,79,91,140
AUC_{ss}[d] [ng h ml^{-1}]	1128±196 (10 mg/d)	830±177 (ER[c] 16 mg/d)	191±140 (cap[c] 12 mg/d), 127±41 (patch 9.5 mg/24h)	386 ± 59 (5mg)	60,79,89,140
CL_{tot} [L h^{-1}]	10±2.5	20±5	120	5-10	81,91,96,140
V [L kg^{-1}]	12 ± 2 L/kg	2.64 L/kg	1.8–2.7 L/kg	4-9	60,70,82,87,91
Metabolism	Hepatic (CYP2D6, CYP3A4, UGT)	Hepatic (CYP2D6, CYP3A4, UGT)	Esterases in liver and intestine	Metabolized to a minor extent	40,62,63,76,82,87
Kinetics	Linear	Linear	Non-linear	Linear	41,58,59,70,89
Steady State	14-21 d	6 d	1 d	11 d	39,59,72,89

a) The new dosage of 23 mg has recently been approved by the FDA for the treatment of moderate to severe AD.
b) The half-life of the drug is short, but the duration of action is longer as the acetylcholinesterase and butyrylcholinesterase are inhibited for 8.5 h and 3.5 h, respectively, through its pseudo-irreversible mechanism.
c) cap=capsule, IR= immediate release, ER=extended release formulation
average±SD.

Chapter 2: Objectives

2.1 General objectives

The objectives of this thesis are to determine pharmacogenetic factors influencing the treatment with antidementia drugs, focusing on drug-metabolizing enzymes and transporters. For this purpose, a clinical study was initiated in collaboration with different psychiatric and geriatric hospitals, outpatient centers and nursing homes in the cantons of Vaud, Geneva and Fribourg. Moreover, analytical methods have to be developed for the measurement of plasma concentrations of antidementia drugs in the study participants and for further use in our TDM service.

2.2 Specific objectives

2.2.1 Analytical methods

The specific aims of the analytical chemistry part of the thesis are:

- to develop a high performance liquid chromatography method with mass spectrometry detection (HPLC-MS) for the plasma level determination of antidementia drugs in the study participants (**Article II**, Chapter 3),
- to transfer the HPLC-MS method to the newly acquired ultra performance liquid chromatography system coupled with tandem mass spectrometry (UPLC-MS/MS) for the use in the routine TDM service of our laboratory (**Article III**, Chapter 3)

2.2.2 Pharmacogenetic study on antidementia drugs

The specific aims of the pharmacogenetic study are:

- to determine genetic factors influencing **donepezil** steady-state plasma concentrations by analysis of polymorphisms in *CYP2D6*, *CYP3A*, *POR* and *NR1I2* genes, and to investigate the relationship of plasma concentrations and the occurrence of side effects (**Article IV**, Chapter 4),
- to determine genetic factors influencing the clearance of **memantine** by a population pharmacokinetic analysis including polymorphisms in genes of organic cation transporters and nuclear receptors and to investigate the relationship of memantine plasma concentrations and the occurrence of side effects (**Article V**, Chapter 4),
- to determine genetic factors influencing **galantamine** steady-state plasma concentrations by analysis of polymorphisms in *CYP2D6*, *CYP3A* and *POR* genes, and to investigate the relationship of plasma concentrations and the occurrence of side effects (**Article VI**, Chapter 4).

2.2.3 Review article

The specific aims of the review article are to perform an extensive literature research on the current knowledge on pharmacodynamics, pharmacokinetics and pharmacogenetics of antidementia drugs and to summarize it for publication in an international journal (**Article I**, Chapter 1).

Chapter 3: Methods

3.1 Clinical Study

A naturalistic cross-sectional study was conducted during the period from December 2008 to March 2011. At different psychiatric and geriatric hospitals, outpatient centers and nursing homes in the cantons of Vaud, Geneva and Fribourg, 303 patients were enrolled receiving for at least one month a treatment with the following antidementia drugs at a stable dose: donepezil (n=129), galantamine (n=27), rivastigmine (n=44) and memantine (n=108). One blood sampling was carried out to measure the steady-state plasma concentration of the drug, to conduct genetic analyses and to determine potential renal and hepatic impairment. Additionally, concomitant diseases, comedication, consumption of grapefruit, alcohol and tobacco and the presence of adverse events related to the antidementia treatment were recorded. The study was approved by the local ethic committees and written informed consent was obtained from all study participants or their legal tutors.

3.2 Analytical Methods

For the quantification of drugs in plasma, reliable and reproducible analytical methods are required. Liquid chromatography coupled to mass spectrometry is the most specific and sensitive technique that is presently used for drug concentration measurements. In this work, a reverse phase HPLC-MS method was developed and subsequently transferred to the newly acquired UPLC-MS/MS system.

HPLC-MS is a powerful technique for the separation and quantification of analytes dissolved in a liquid. The injected sample is transported by the mobile phase through a column containing the stationary phase. Through interaction with the stationary phase, the analytes are retained on the column and, depending on their chemical properties, are eluted from the column at characteristic retention times. The analytes are conducted to the MS detector, where they are ionized in the source and analyzed in an electromagnetic field according to their mass-to-charge ratio. The UPLC-MS/MS technology offers several benefits compared to HPLC-MS. A shortened run time and a higher resolution are achieved by the use of smaller columns packed with sub-2-µm particles in combination with a system that allows to work at high pressures and with low dispersion. Moreover, the MS/MS detector increases the specificity of the method. Since two mass spectrometer selections are involved in the detection, separated by a collision cell where a fragmentation of the molecule takes place, the MS/MS determines specifically the mass of the analyte and its characteristic fragment.

To analyze drugs in plasma, a sample clean-up is needed prior to injection into the chromatography system. Through the sample preparation, interfering substances as well as particles or proteins that could precipitate in the system are removed from the matrix, thus, the quality of the results are improved, and the instrument performance and column life time are prolonged. In this work, a sample

preparation by solid phase extraction (SPE) was used for the HPLC-MS procedure, whereas the increased specificity allowed a simple protein precipitation for the UPLC-MS/MS procedure.

It is important that the applied bioanalytical methods are well characterized and fully validated. The objective of a method validation is to give guarantees that measurements performed in routine will be close to the unknown true value of the sample and inside acceptance limits that were set according to the intended application of the method.[60] In this work, the method validations were conducted in accordance with the international guidelines of the FDA, the EMEA and the "Société Française des Sciences Techniques Pharmaceutiques".[60-62] The decision to accept a bioanalytical method is based on different validation parameters, including accuracy, precision, selectivity, matrix effects and stability of the compounds in the biological matrix and in the working solutions.
Once the analytical method has been validated, its accuracy and precision should be regularly monitored to ensure that the method continues to perform satisfactorily during routine use. Therefore, internal quality control samples are added to each run of patients' samples and external quality control samples are analyzed several times per year.

Details of the development and validation of the HPLC-MS and UPLC-MS/MS procedures for the simultaneous determination of antidementia drugs are described in Article II and III (Chapter 3.4 and 3.5).

3.3 Genetic Methods

In this work, the patients were genotyped for selected SNPs by real-time polymerase chain reaction (PCR) with 5'-nuclease allelic discrimination assays, also known as TaqMan SNP genotyping assays.

With the PCR technique, a selective enrichment of a specific DNA sequence by a factor of one million is feasible.[63] PCR amplification involves two oligonucleotide primers that flank the DNA segment to be amplified and repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences and extension of the primers with a thermostable DNA polymerase.[63] Since the two resulting extension products are also complementary to and capable of binding primers, each successive cycle essentially doubles the amount of DNA synthesized in the previous cycles, leading to an exponential accumulation of the specific target fragment over the approximately 40 cycles used in a PCR reaction.[63]

For genotyping with the TaqMan technique, two differentially labeled fluorescent TaqMan probes are added to the PCR reaction, that are designed as perfect matches to either of the two allelic variants (Figure 7).[64] During each amplification cycle, the TaqMan probes hybridize to its complementary target region and, during the primer extension step, is cleaved by the 5'-nuclease activity of the DNA polymerase. Thereby, the quencher and fluorescent reporter are separated. As a result, the reporter

fluorescent signal can be measured quantitatively for each of the two allele-specific TaqMan probes. The fluorescent intensity increases with each subsequent amplification cycle.[64]

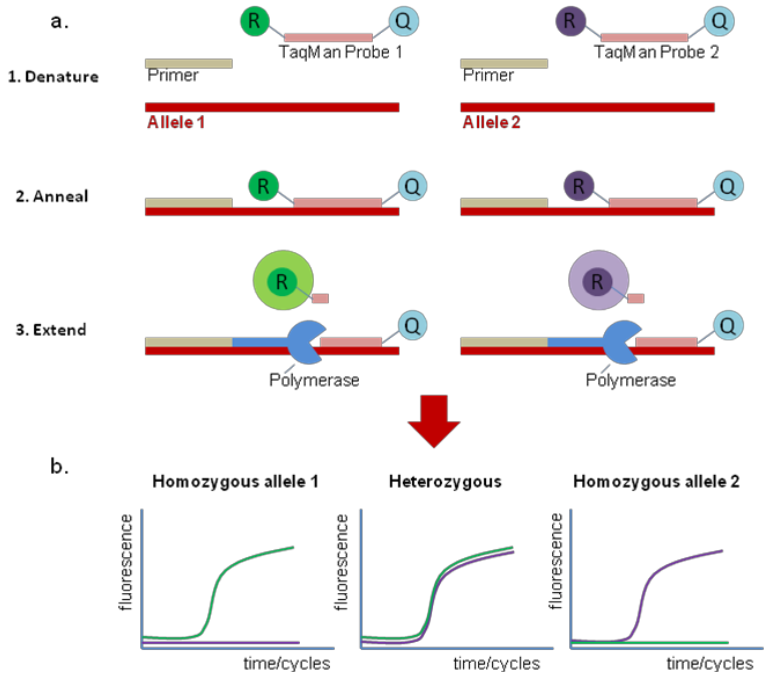

Figure 7: a. The 5'-nuclease activity of the polymerases used in the PCR reaction cleaves the TaqMan probes during the amplicon extension step, which separates the detecable reporter fluorophore (R) from the quencher (Q). **b.** The emitted fluorescence is measured at each PCR cycle for the two TaqMan probes. Homozygous subjects for the allele 1, heterozygous subjects and homozygous subjects for the allele 2 can be differentiated. Picture adapted from Koch WH, Nat Rev Drug Discov, 2004;3(9):749-61.

CYP2D6 gene deletion or multiplication (*CYP2D6*5 and CYP2D6*xN*) was determined by the CYP2D6 TaqMan copy number assay. For this analysis, two labeled TaqMan probes are used, one probe detects a sequence in the target gene, whereas the other probe detects a sequence on a gene that is known to exist in only two copies in the genome. By relative quantification of the emitted fluorescence, the number of *CYP2D6* gene copies is assigned. Moreover, the presence of gene multiplication was confirmed by long PCR analyses followed by amplicon separation through gel electrophoresis, according to the method of Løvlie et al.[65] In patients with multiple gene copies, an additional amplicon with a specific length is detected, corresponding to a sequence of the *CYP2D6-CYP2D6* intergenetic region.

3.4 Article II: Simultaneous determination of antidementia drugs in human plasma for therapeutic drug monitoring

Summary

In this article, an HPLC-MS method for the quantification of antidementia drugs in human plasma is described. The analytical method allows the simultaneous determination of donepezil, galantamine, rivastigmine and its major metabolite NAP 226-90, and memantine at therapeutic concentrations, with lower limits of quantifications at 1 ng/mL or 2 ng/mL. The analytical procedure met the validation criteria of international guidelines and showed to be reliable and reproducible in the following analyses. It was successfully applied to the plasma samples of the 300 participants of the pharmacogenetic study as well as to external quality control samples and samples of other laboratories with known concentrations.

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SIMULTANEOUS DETERMINATION OF ANTIDEMENTIA DRUGS IN HUMAN PLASMA FOR THERAPEUTIC DRUG MONITORING

Muriel Noetzli[1], Eva Choong[1], Nicolas Ansermot[1], Chin B. Eap[1,2]

1. Unit of Biochemistry and Clinical Psychopharmacology, Centre for Psychiatric Neuroscience, Department of Psychiatry, CHUV, University of Lausanne, Hospital of Cery, Prilly, Switzerland.
2. School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Boulevard d'Yvoy 20, 1211 Geneva 4, Switzerland.

Author contributions

Concept: C.B. Eap

Method development: M. Noetzli

Scientific inputs for the method development: all authors

Drafting of the manuscript: M. Noetzli

Critical revision of the manuscript for important intellectual content: all authors

Abstract

Backgrounds: A simple liquid chromatography mass spectrometry method was developed and validated for the simultaneous determination of antidementia drugs including donepezil, galantamine, rivastigmine and its major metabolite NAP 226-90, and memantine.

Methods: A solid phase extraction procedure with a mixed-mode sorbent was used to isolate the drugs from 0.5 mL human plasma. Reverse-phase chromatographic separation of the compounds was obtained with a gradient elution of an ammonium acetate buffer at pH 9.3 and acetonitrile and the analytes were detected by mass spectrometry in the single ion monitoring mode.

Results: The method was validated according to the recommendations of the FDA, including assessment of trueness (-8.0 to +10.7 %), precision (repeatability: 1.1 to 4.9%, intermediate precision: 2.1 to 8.5 %), selectivity and matrix effects variability (<6%), as well as short- and long-term stability in plasma. The calibrated ranges were comprised between 1 ng/mL to 300 ng/mL (rivastigmine and memantine) and 2 ng/mL to 300 ng/mL (donepezil, galantamine and NAP 226-90).

Conclusions: The method was successfully applied to patients' samples and might contribute to evaluate whether a Therapeutic Drug Monitoring (TDM) guided dose adjustment of antidementia drugs could contribute to minimize the risk of adverse reactions and to increase the probability of efficient therapeutic response.

1. Introduction

Alzheimer's disease (AD) is the most frequent type of dementia among elderly people. It is characterized by a progressive cognitive and functional decline associated with neuropathologic lesions and neurochemical alterations such as a cholinergic deficit and glutamatergic overstimulation of postsynaptic N-methyl-D-aspartate (NMDA) receptors. The neurochemical changes in AD are the basis for the symptomatic treatment with the four drugs currently used. Donepezil, galantamine and rivastigmine are inhibitors of the acetylcholinesterase, increasing the concentration and duration of action of acetylcholine in the brain, whereas memantine, an NMDA-antagonist, reduces the glutamatergic overstimulation.[1,2] The chemical structures of the four drugs and the major metabolite of rivastigmine, NAP 226-90, are presented in Fig. 1.

Even though the benefit of Therapeutic Drug Monitoring (TDM) of antidementia drugs still remains to be demonstrated, several factors indicate that TDM might be useful for these drugs.[3] Patients taking antidementia drugs show a high inter-individual variability in their response to treatment,[4-6] which might partly be due to the observed inter-individual differences in plasma concentrations triggered by variations in drug metabolism and elimination.[7-9] Donepezil and galantamine are metabolized by the cytochrome P450 3A (CYP3A) and 2D6 (CYP2D6) enzymes, known for their high inter-individual differences in activity[10-12]. Genetic variations in the CYP2D6 have been shown to influence plasma levels of these two drugs.[7,13] In contrast, variability in renal elimination accounts probably for the inter-

individual differences in plasma concentrations of memantine, since it is mainly excreted unchanged by the kidneys.[14] It is supposed that genetic variations in transporters involved in the active tubular secretion of the drug could have an impact on its plasma levels.[15,16] Rivastigmine is extensively metabolized by cholinesterases to NAP 226-90, an inactive and non-toxic metabolite. However, this inactive metabolite is an indicator of the extent of metabolism of rivastigmine, in particular of the first pass effect, and therefore it might be beneficial to quantify NAP 226-90 in the plasma.[17] Moreover, comorbidities, primarily renal and hepatic impairment, and polymedication leading to drug interactions, make elderly people susceptible to changes in plasma levels of the drugs. All these factors let assume that an individually adapted dosage through monitoring of the plasma concentrations might be beneficial for patients treated with antidementia drugs. Furthermore, non-adherence to the therapy due to the decline in cognitive function is a particular problem in geriatric patients.[18] Detected by TDM, this problem could be overcome by supervision of drug intake.

Up to now, no method has been published that allows the simultaneous quantification of the four commonly used antidementia drugs in plasma. However, different chromatographic methods are described to determine the single compounds in human blood, sometimes with their related metabolites. Although some methods are described using gas chromatography,[19,20] the majority uses liquid chromatography coupled with tandem mass spectrometry,[21-31] ultraviolet (UV)[32,33] or fluorescence detection.[12] In case of mass spectrometry (MS), the analysis is mostly done by electrospray in the positive mode (ESI+)[21,23,26,27] rather than by atmospheric pressure chemical ionization.[22,31] Several different extraction procedures are described in the literature comprising of liquid-liquid extraction,[26,32] solid phase extraction (SPE)[21,24,33] and protein precipitation.[23] Moreover, one capillary electrophoresis method using UV detection has been published, describing the simultaneous determination of galantamine, rivastigmine and NAP 226-90 for drug monitoring.[34] The use of isotope-labeled internal standards to increase the robustness of the method is described in two of the published methods.[25,29] Since most of these analytical methods are developed for pharmacokinetic studies after single doses,[21,22,26-28,32,33] the covered concentration ranges are narrow and would not be adequate for TDM in steady state conditions of different dosages, where a large range of plasma concentrations is expected.

The objective of the present work was to develop and validate a rapid and sensitive LC-MS method for the simultaneous determination of antidementia drugs in human plasma for the routine use in a TDM laboratory. The compounds were detected by MS in ESI+ mode, which is reported to be sensitive to matrix effects.[35,36] Therefore, a mixed-mode cation exchange SPE, a powerful clean-up procedure, was chosen to isolate the drugs from the plasma prior to analysis. Furthermore, for three of the five compounds isotope-labeled standards were used for quantification, known to effectively normalize matrix effects.[37] The method was successfully validated according to the guidelines of the Food and Drug Administration (FDA) and applied to samples of a real population treated with antidementia drugs.

2. Experimental

2.1 Chemicals and reagents

The drugs were kindly provided by their manufacturers: donepezil HCl by Eisai Co., Ltd (Tokyo, Japan), galantamine HBr by Janssen-Cilag (Beerse, Belgium), memantine HCl by Merz (Frankfurt/Main, Germany), rivastigmine hydrogen tartrate and its metabolite NAP 226-90 by Novartis (Basel, Switzerland). The internal standards (IS) [$^{2}H_{7}$]-donepezil, [^{13}C,$^{2}H_{3}$]-galantamine HCl, [$^{13}C_{2}$,$^{2}H_{6}$]-memantine HCl and [$^{2}H_{6}$]-rivastigmine hydrogen tartrate (not used in the final method) were purchased from Alsachim (Strasbourg, France). Lichrosolv® HPLC-grade acetonitrile, hydrochloric acid (37%) and ortho-phosphoric acid (85%) were purchased from Merck (Darmstadt, Germany). Ammonium hydroxide (25%), ammonium acetate for MS, formic acid for MS and physostigmine hemisulfate (eserine) were bought from Sigma-Aldrich (Steinheim, Germany). Ultrapure water was obtained from a Milli-Q® RG with a QPAQ2 column system (Millipore, Billerica, MA, USA). All chemicals were of analytical grade. For the preparation of calibration standards (CS) and quality control (QC) samples and the evaluation of matrix effects, more than 10 different batches of human plasma from outdated blood donation units were obtained from the Hospital's blood transfusion center (CHUV, Lausanne, Switzerland).

2.2 Equipment

The liquid chromatography system consisted of an Agilent Series 1100 LC equipped with a binary pump and a 100-vial autosampler (Agilent Technologies, Waldbronn, Germany), with a measured dwell volume of 1.05 mL. Data handling and instrument control were performed by the Chemstation software B.01.01 (Agilent Technologies). The chromatographic system was coupled to an Agilent Series 110 MSD single quadrupole mass spectrometer (Agilent Technologies) equipped with an electrospray ionization interface operated in positive ionization mode (ESI+). Chromatographic separations were performed on an XBridge C18 column (2.1 x 100 mm; 3.5 µm) (Waters, Milford, MA, USA) equipped with an XBridge C18 cartridge (2.1 x 10 mm; 3.5 µm). Analyses were carried out in an air conditioned room at 22°C.

2.3 Stock and working solutions

Stock solutions of the analytes were prepared at 1 mg/mL (as base) by dissolving the adequate amount of the pure analyte in methanol and were stored at -20°C. By dilution of the stock solutions with 0.01 N HCl, working solutions at 100 µg/mL were obtained and were stored at -20°C. Calibrators (CS) and quality control (QC) samples were prepared independently by spiking blank plasma at different concentrations with freshly made dilutions of the working solution at 0.1, 1 and 10 µg/mL. CS and QC were analyzed immediately or stored at -20°C until analysis. The stock solutions of the IS were prepared at 1 mg/mL in methanol and stored at -20°C. They were combined to give a single IS working solution at 0.5 µg/mL that was likewise stored at -20°C.

To competitively inhibit the enzymatic *in-vitro* hydrolysis of rivastigmine to its metabolite NAP 226-90 by plasma cholinesterases, 50 µL of a 0.002 *M* physostigmine solution was added to every 1 mL of plasma and used for the preparation of CS and QC samples.[29]

2.4 Sample preparation

Plasma samples (500 µL) were mixed with 50 µL of IS solution and diluted with 500 µL of a 4% ortho-phosphoric acid solution. 1000 µL of the mixture were loaded onto an Oasis MCX 10 mg 96-well extraction plate (Waters, Milford, MA, USA) previously conditioned and equilibrated with 500 µL acetonitrile and 500 µL water. Subsequently, two washing steps were performed with 500 µL of a 2% formic acid solution followed by 500 µL acetonitrile. The compounds were then eluted with 500 µL acetonitrile/ammonium hydroxide 25% (80:20, v/v). After each step a low vacuum was applied until the wells were dry. The extracted samples were evaporated (N_2 flow, 40°C) and reconstituted in 250 µL of the mobile phase at initial conditions, namely 50 mM ammonium acetate at pH 9.3 and acetonitrile (80:20, v/v), prior to injection into the chromatographic system.

2.5 LC-MS conditions

To set up the parameters for chromatographic separation, the HPLC modeling software OSIRIS Version 4.1 (Datalys, Grenoble, France) was used. The most suitable separation was achieved at a flow rate of 0.3 mL/min with a mobile phase composed of 50 mM ammonium acetate in ultrapure water adjusted to pH 9.3 (solution A) and acetonitrile (solution B) using the following stepwise elution program with an overall run time of 15 min: 20% of B maintained for 1 min, 33% of B at 1.4 min, 41% of B at 2.4 min, 56% of B at 4.2 min and a hold of 80% of B from 4.9 min to 6.5 min. The gradient was followed by rinsing with 90% of B from 6.7 to 8.2 min and a reconditioning step at the initial conditions from 9.2 to 15 min. Of each sample 5 µL were injected.

Detection was performed in the selected ion monitoring (SIM) mode for the singly charged positive ions at *m/z* 380.1 (donepezil), 288.1 (galantamine), 206.1 (rivastigmine), 121.1 (NAP 226-90), 180.1 (memantine), 387.2 [$^{2}H_{7}$]-donepezil, 292.1 [$^{13}C,^{2}H_{3}$]-galantamine and 188.2 [$^{13}C_{2},^{2}H_{6}$]-memantine. Nitrogen was used as nebulizing gas at 20 psi and as drying gas at a temperature of 350°C and a flow rate of 12 L/min. Capillary voltage was set at 1250 V and the fragmentor voltage at 150 V, 130 V, 160 V, 140 V and 100 V for donepezil, galantamine, rivastigmine, NAP 226-90 and memantine, respectively. Dwell time for each ion was 24 ms. In Table 1 the optimized individual fragmentor settings are listed together with the *m/z*-ratios of the compounds.

2.6 Method validation

The method validation was based on the guidelines for Bioanalytical Method Validation published online by the FDA,[38] as well as on the recommendations of the “Société Française des Sciences et Techniques Pharmaceutiques”.[39] The draft version of the guidelines for method validation of the European Medicines Agency (EMEA) was also taken into consideration.[40]

2.6.1 Selectivity, carry-over and psychiatric co-medication

Method selectivity was ascertained by analyzing 10 blank plasma batches for interfering peaks at retention times of the analytes. Possible carry-over effects were assessed by injecting blank plasma samples after the highest calibration standard at 300 ng/mL.

Additionally, the following psychiatric drugs, and some of their metabolites, were extracted and analyzed with the same method to investigate the influence of potential co-medication: amitriptyline, amisulpride, aripiprazole, atomoxetine, dehydro-aripiprazole, bupropion, 3-hydroxy-bupropion, caffeine, chlorpromazine, citalopram, desmethyl-citalopram, clomipramine, desmethyl-clomipramine, clopenthixol, clozapine, N-oxid-clozapine, norclozapine, desipramine, duloxetine, fluoxetine, norfluoxetine, flupenthixol, fluvoxamine, haloperidol, imipramine, loxapine, maprotiline, mianserin, desmethyl-mianserin, midazolam, 1-hydroxy-midazolam, mirtazapine, 8-hydroxy-desmethyl-mirtazapine, moclobemide, N-oxid-moclobemide, 3'-oxo-moclobemide, morphine, nicotine, nortriptyline, olanzapine, paroxetine, quetiapine, reboxetine, risperidone, 9-hydroxy-risperidone, sertindole, dehydro-sertindole, sertraline, desmethyl-sertraline, sulpiride, trimipramine, desmethyl-trimipramine, trazodone, venlafaxine, O-desmethyl-venlafaxine, N-desmethyl-venlafaxine, N-O-di-desmethyl-venlafaxine and ziprasidone. In case of similar retention times as the antidementia drugs, suppression of the signal was assessed by comparing peak area of the analyte at 100 ng/ml alone with the peak area of the analyte when injected with the potential interfering compound at a high therapeutic concentration.

2.6.2 Matrix effects and recovery

Primarily, matrix effects were investigated qualitatively by simultaneous post-column infusion of the analytes and IS into the MS detector during chromatographic analyses of 6 different blank plasma extracts, water and mobile phase.[35,41] The analytes and their IS were infused at a concentration of 1 µg/mL and a flow rate of 2 µL/min. The chromatographic signal of each analyte was carefully examined to ascertain that there is no signal suppression or enhancement at the retention time due to interfering substances.

Subsequently, matrix effects and recovery were assessed quantitatively at low (10 ng/mL) and high concentration levels (150 ng/mL for donepezil, rivastigmine and NAP 226-90, 300 ng/mL for galantamine and memantine) and at 50 ng/mL for the IS based on the approach of Matuszewski.[42] The following three sets of samples were prepared:

(A): Pure standard solution samples of the analytes and IS in the SPE reconstitution solvent (acetonitrile/buffer 20:80) injected directly onto the column

(B): Duplicates of plasma extract samples from 6 different sources spiked with the analytes and IS after extraction

(C): Duplicates of plasma samples from 6 different sources (same as B) spiked with the analytes and IS before extraction.

The matrix effect (ME) was evaluated for each analyte and IS by calculating the ratio of the peak area in the presence of the matrix (samples spiked after extraction) to the peak area in absence of the matrix (pure standard) and expressed in percentage (ME = B/A). The recovery of extraction (RE) in the matrix was determined by comparing the peak area of the pre-extraction spiked (C) to the post-extraction spiked samples (B) (RE = C/B). The overall process efficiency (PE), taking into account ME and RE, was assessed by calculating the ratio of the peak area of the pre-extraction spiked samples to the peak area of the pure standard (PE = C/A). Of all three parameters, the variability between the different plasma batches was evaluated and expressed as coefficient of variation (CV %).

2.6.3 Trueness and precision

Three validation series were carried out to examine trueness and precision of the method. CS were set in duplicate at 4 levels and QC samples in quadruplicate at 8 different levels covering the expected range of concentrations in patients.[9,11,17,43] CS 1, 20, 100, 300 ng/mL for memantine and rivastigmine and 2, 20, 100, 300 ng/mL for donepezil, galantamine and NAP 226-90; QC 2, 5, 20, 50, 100, 200, 300 ng/mL for donepezil, 1, 2, 5, 20, 50, 100, 200, 300 ng/mL for memantine and rivastigmine and 2, 4, 10, 20, 50, 100, 200, 300 ng/mL for galantamine and NAP 226-90. Calibration curves were freshly prepared and established for each batch considering the peak area ratio of the analytes and their IS. [$^{2}H_{7}$]-donepezil, [^{13}C,$^{2}H_{3}$]-galantamine and [$^{13}C_{2}$,$^{2}H_{6}$]-memantine were used as IS for their target analyte, whereas [^{13}C,$^{2}H_{3}$]-galantamine was also found to be a suitable IS for rivastigmine and NAP 226-90. The QC samples were analyzed against the calibration curve of the same run and the trueness of the obtained value was expressed as percentage of the nominal value. Repeatability (intra-day variance) and intermediate precision (sum of intra-day and inter-day variances) were calculated and expressed as coefficients of variation (CV %).[44] In accordance to the above-mentioned guidelines, the lower limit of quantification (LLOQ) for each analyte was determined by the lowest QC concentration with a trueness and precision of ≤20%.

To establish a procedure if patients' plasma levels exceed the concentration of the highest calibration standard at 300 ng/mL, the trueness and precision of a three-fold dilution of spiked plasma at 600 ng/mL was assessed. The dilution was carried out with blank plasma.

2.6.4 Stability

Stability of the compounds in plasma was assessed by spiking 5 different blank plasma batches at low (2ng/mL for memantine and rivastigmine, 4 ng/mL for donepezil, galantamine and NAP 226-90) and high concentration (250 ng/mL). Different sets of aliquots were quantified after storage at ambient temperature and at 5°C up to 72 hours, at -20°C for 3 months, and after one to three freeze-thaw cycles. The variations in drug concentrations were expressed as percentage of the initial concentration found in the samples analyzed immediately after preparation.

Furthermore, the post-preparative stability was assessed by leaving the processed samples up to 24 h at room temperature as well as 48 h at 5°C before reanalysis. The calculated concentrations after immediate analysis and after storage of the same samples were compared.

Finally, the stability in spiked whole K-EDTA blood was tested at 100 ng/mL for donepezil, galantamine and memantine and at 10 ng/mL and 250 ng/mL for rivastigmine and NAP 226-90 by leaving the samples up to three days at room temperature. To stop the *in vitro* degradation of rivastigmine by plasma cholinesterases, 10 µL of a physostigmine solution at 0.01 *M* was added to every 1 mL of blood.

3. Results and Discussion

3.1 Solid-phase extraction

To extract the basic drugs from the plasma, a solid phase extraction procedure with a mixed-mode sorbent was chosen, since the combination of hydrophobic interactions and cation-exchange leads to an enhancement of selectivity. The use of different organic solvents as well as different washing steps was investigated. Satisfactory results regarding recovery, repeatability and selectivity were obtained by using a generic protocol with acetonitrile as organic solvent and formic acid as washing solution. The recoveries, presented in Table 2, were comprised between 88 and 98% with good repeatability (CVs between 1 and 6%). By evaporation of the elution solvent and reconstitution of the drugs in the mobile phase, a twofold concentration step was performed, allowing the quantification of lower plasma concentrations. Moreover, the peak shape was thereby improved compared to the injection of the pure elution solvent or the elution solvent diluted with water (50:50). The selected SPE procedure is rapid and could be easily automatable, using 500 µL of plasma.

3.2 Optimization of chromatographic and MS conditions

Different mobile phase compositions were assessed on reverse phase columns using acetonitrile or methanol and buffers ranging from pH 3 to pH 10. For the studied drugs, acidic conditions were less suitable than basic conditions, as the more polar bases, namely NAP 226-90 and galantamine, were eluted rapidly therefore increasing the risk of interferences with unretained matrix components. Moreover, the peak shapes were better in basic conditions. These observations are in agreement with literature reports.[45,46] The most suited chromatographic separation of the five compounds was achieved by a gradient elution using as mobile phase an ammonium acetate buffer 50 mM at pH 9.3 and acetonitrile as organic solvent. As stationary phase, an XBridge C18 column was selected due to the satisfactory selectivity results and its stability under basic conditions. Three different concentrations of ammonium acetate buffer (20 mM, 50 mM and 100 mM) were tested. Interestingly, the concentration of the buffer had an important influence on the selectivity with the most satisfactory result at 50 mM. In addition, the higher concentration of ammonia acetate increased the reproducibility of the chromatographic separation with increasing age of the column. With the selected LC conditions, the overall run time was 15 min. The typical retention times of the analytes are listed in Table 1 and a chromatogram of a QC plasma sample of the 5 compounds and the IS at 50 ng/mL is shown in Fig. 2.

The use of pure isotope-labeled internal standards is highly recommended in LC-MS analysis in order to compensate signal suppression or enhancement due to matrix components and variability in extraction procedure.[37,47] In the described method the isotope-labeled IS [$^{2}H_{7}$]-donepezil, [$^{13}C,^{2}H_{3}$]-galantamine and [$^{13}C_{2},^{2}H_{6}$]-memantine were used for quantification of their corresponding co-eluting analytes, whereas [$^{13}C,^{2}H_{3}$]-galantamine was also chosen as IS for rivastigmine and NAP 226-90 for the following reasons: the fragment ions of rivastigmine and NAP 226-90 were used for quantification as they had a higher intensity than the molecule ions, allowing an increase in sensitivity for the two compounds. Since [$^{2}H_{6}$]-rivastigmine and rivastigmine have the same fragment ion at *m/z* 206, the deuterated standard couldn't be used as IS for rivastigmine. Moreover, for the metabolite of rivastigmine, NAP 226-90, no isotope-labeled standard was commercially available. Thus, after having verified that there is no signal suppression from galantamine on [$^{13}C,^{2}H_{3}$]-galantamine at the highest CS at 300 ng/mL, [$^{13}C,^{2}H_{3}$]-galantamine was used as IS for rivastigmine and NAP 226-90. The following MS settings were evaluated to find the optimal ionization conditions: nebulizing gas pressure (20-60, selected 20 psi), drying gas temperature (200-350, selected 350°C), drying gas flow rate (7-13, selected 12 L/min) and capillary voltage (1250-4000, selected 1250 V).

3.3 Validation

3.3.1 Selectivity, carry-over and psychiatric co-medication

No peaks from endogenous compounds were observed at the drugs retention time in any of the 10 blank plasma extracts evaluated. However, a slight carry-over effect of 0.1% for donepezil was observed. With the highest CS at 300 ng/mL, this memory effect is acceptable as it corresponds to less than 20% of the concentration at LLOQ. For the other compounds, no carry-over was detected.

Due to several co-morbidities, patients taking antidementia drugs often receive multiple co-medications that could potentially lead to analytical interferences. Therefore, the influence of possible psychiatric co-medication on the determination of plasma levels of antidementia drugs was assessed. For this purpose, several antidepressant and antipsychotic drugs, and some of their metabolites, were extracted with the same method and their retention time was recorded. Four substances co-eluted with the compounds of interest, namely donepezil with bupropion, rivastigmine with risperidone, and memantine with 3-hydroxy-bupropion and 9-hydroxy-risperidone. However, they were distinguished by MS detection and no clinically significant signal suppression was observed for the antidementia drugs when injected with these compounds. Since the co-medication of elderly patients is not restrained to psychiatric drugs, the present assessment of interferences is not exhaustive and during routine use of the method, special attention should be paid to this issue. Nevertheless, it is supposed that, for donepezil, galantamine and memantine, the co-eluting isotope-labeled internal standards normalize the signal suppression.

3.3.2 Matrix effects

By means of direct infusion of the drug substances and the IS into the MS detector during analysis of 6 different blank plasma batches, qualitative signal suppression and enhancement was assessed. No

interferences were apparent at the retention times of the analytes and IS. In Fig. 3 the corresponding chromatograms are presented.

In the quantitative assessment of interfering plasma components, slight matrix effects were observed ranging from 85 to 114%. When normalized by their co-eluting isotopic-labeled IS, the matrix effects were considerably smaller ranging from 91 to 104% (data not shown). However, even more important than having low matrix effects, is having a low variability of these effects between the different plasma batches. With the selected extraction procedure this aim was achieved, as the variability between the 6 different plasma sources never exceeded 5%. The results are reported in Table 2.

Finally, the process efficiencies, describing the overall recovery taking the extraction recovery and the matrix effects into account, were comprised within 82 and 111% with CVs ranging from 1 to 6%.

3.3.3 Trueness and precision

Three validation series were performed on three different days. Eight CS were used for each compound covering the range from 1 to 300 ng/mL for donepezil, memantine and rivastigmine and from 2 to 300 ng/mL for galantamine and NAP 226-90. Different calibration curve models were tested and the following four point calibration curves were selected: 1, 20, 100 and 300 ng/mL for rivastigmine and memantine using a quadratic regression model and a linear regression weighted by 1/x, respectively; 2, 20, 100, 300 ng/mL for donepezil, NAP 226-90 and galantamine using a quadratic regression model for donepezil and linear regression models for NAP and galantamine weighted by $1/x^2$ and 1/x, respectively.

The trueness, repeatability and intermediate precision of the back calculated QC samples are reported in Table 3. For all QC samples, the determined trueness met the acceptance criterion of ±15% (LLOQ ±20%); except for donepezil at 1 ng/mL the trueness was 128%. However, the criterion for donepezil was met at 2 ng/mL. Overall, the repeatability and intermediate precision did not exceed the required limit of ≤15% (LLOQ ≤20%). Consequently, the LLOQ was set at 1 ng/mL for rivastigmine and memantine and at 2 ng/mL for donepezil, NAP 226-90 and galantamine. For rivastigmine and its metabolite a low LLOQ is mandatory given the short half life of the drug.[17] By contrast, for the other three drugs the LLOQ is well below the expected therapeutic concentrations of patients. The corresponding accuracy profiles with acceptance limit (λ= $\pm$30%), and with upper and lower β-expectation tolerance intervals (β = 90%) calculated for each compound in the dosing range are shown in Fig. 4.

Three-fold dilutions of spiked plasma at concentrations exceeding by two-fold the highest calibration levels were found to be in the accepted range of the accuracy profile (not shown in Fig. 4). This indicates that plasma samples containing antidementia drugs above the highest level of calibration can be adequately diluted with blank plasma prior to LC-MS analysis.

To assess linearity of the method, a linear regression model was applied to the recalculated QC concentrations *vs.* theoretical concentrations. The following slopes 0.983, 0.983, 1.024, 0.986 and

0.990, and intercepts 0.389, 1.745, -0.721, 2.302 and 1.445 were found for donepezil, galantamine, rivastigmine, NAP 226-90 and memantine, respectively. The corresponding determination coefficients were 0.991, 0.998, 0.996, 0.995, 0.999, indicating that the developed method was linear for the tested compounds.

3.3.4 Stability

As reported in Table 4, the stability of the analytes in plasma was confirmed at room temperature and at 4°C up to 72 h as well as at -20°C for three months. The variation over time of each analyte was well within the required range of ±15% of the initial concentration. Furthermore, all of the analytes were found to have a good stability in plasma even after three freeze and thaw cycles. The stability of the compounds in the extracted samples was ascertained by leaving the vials for 24 h at room temperature and for 48 h at 5°C.

To establish shipping conditions, the stability of the drugs in whole K-EDTA blood left at room temperature up to three days was investigated. The stability up to 72 h was confirmed for donepezil and galantamine, as well as for rivastigmine when physostigmine was immediately added to the blood samples to inhibit enzymatic breakdown. In contrast, NAP 226-90 was found to be unstable in whole blood with a mean decrease for the high concentration of -15% after 8 h and -38% after three days. For the determination of the metabolite NAP 226-90, a rapid centrifugation is necessary to stop the degradation process. Interestingly, for memantine an increase of 17% and 20% was observed after two and three days, respectively. Therefore, a centrifugation step within 24 h is preferable for the precision of the measurement. A possible explanation of this raise could be the hemolysis of blood cells during storage and therefore a release of drug substance into the plasma. A deeper investigation of this phenomenon would be outside the scope of the present work.

4. Clinical Application

The presence of several comorbidities, drug interactions due to polypharmacy, and variations in drug metabolism are possible factors leading to the high inter-individual variability in plasma levels observed in patients treated with antidementia drugs. Since a lack of clinical response or the presence of adverse events could be due to non-optimal plasma levels of the drugs, TDM might be a valuable tool to individually adapt the dosage of antidementia drugs. The developed analytical method showed to be reliable and sensitive for monitoring plasma concentrations of antidementia drugs. It is currently applied to samples from participants of a pharmacogenetic study investigating the influence of genetic variations in drug metabolizing enzymes and drug transporters on the plasma concentrations of antidementia drugs. Some examples of measured plasma levels are shown in Table 5. Additionally, a representative patient's chromatogram for each drug is shown in Fig. 5.

5. Conclusion

A simple LC-MS method for the simultaneous quantification of donepezil, galantamine, rivastigmine and its metabolite, and memantine was developed and validated according to the FDA guidelines. The drugs were extracted from plasma by a simple SPE procedure, hereby removing efficiently endogenous interfering components from the matrix. The HPLC-MS method allows a fast quantification of the five compounds over a concentration range usually measured in patients, which was confirmed by applying the method to real patients' samples.

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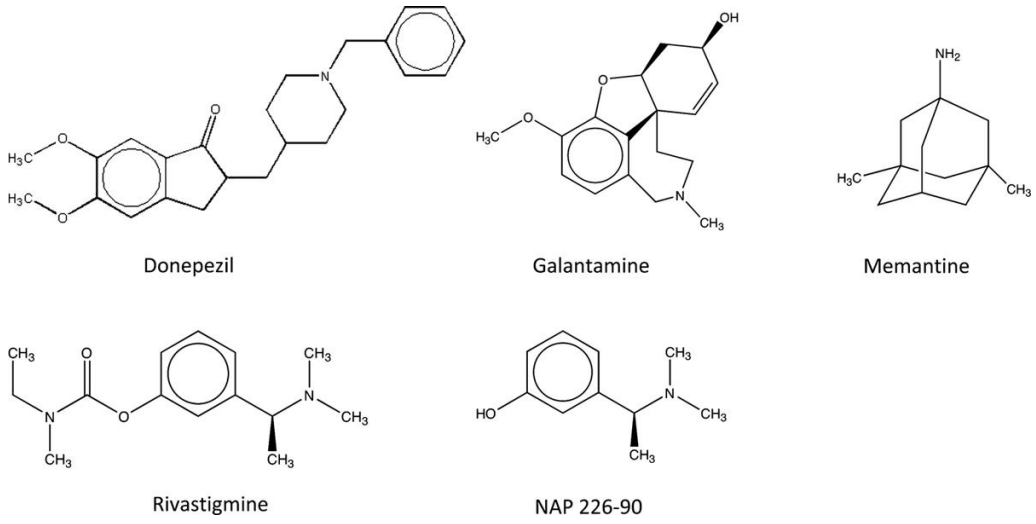

Figure 1: Chemical structures of the antidementia drugs and the metabolite NAP 226-90.

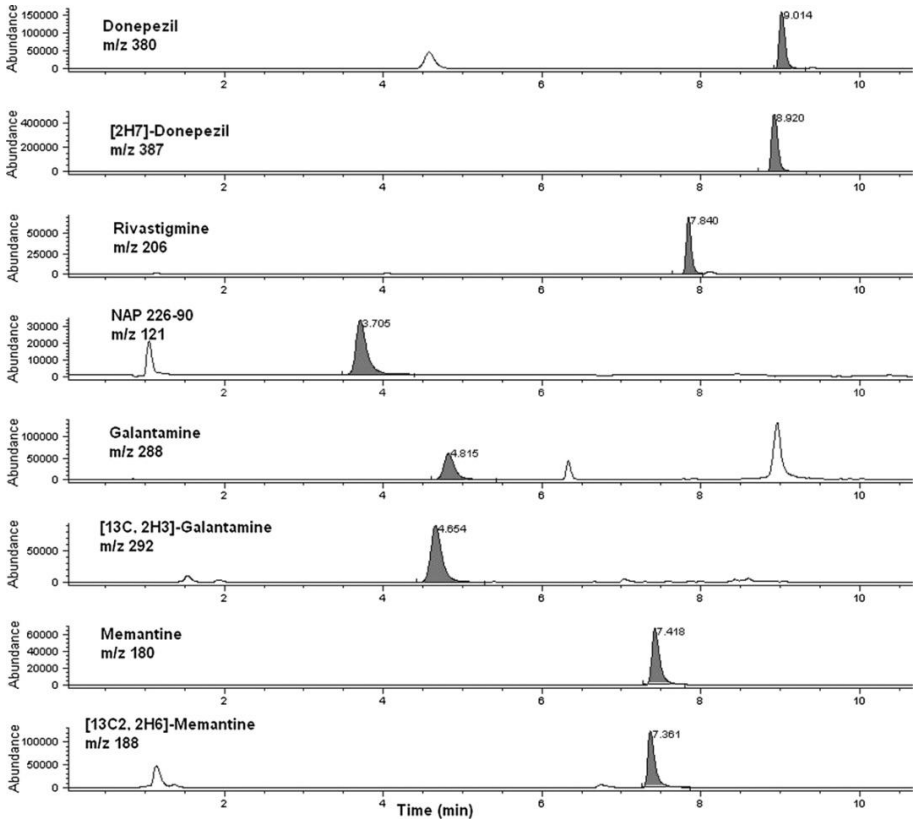

Figure 2: Selected ion monitoring chromatogram of a QC plasma sample at 50 ng/ml for all compounds and IS.

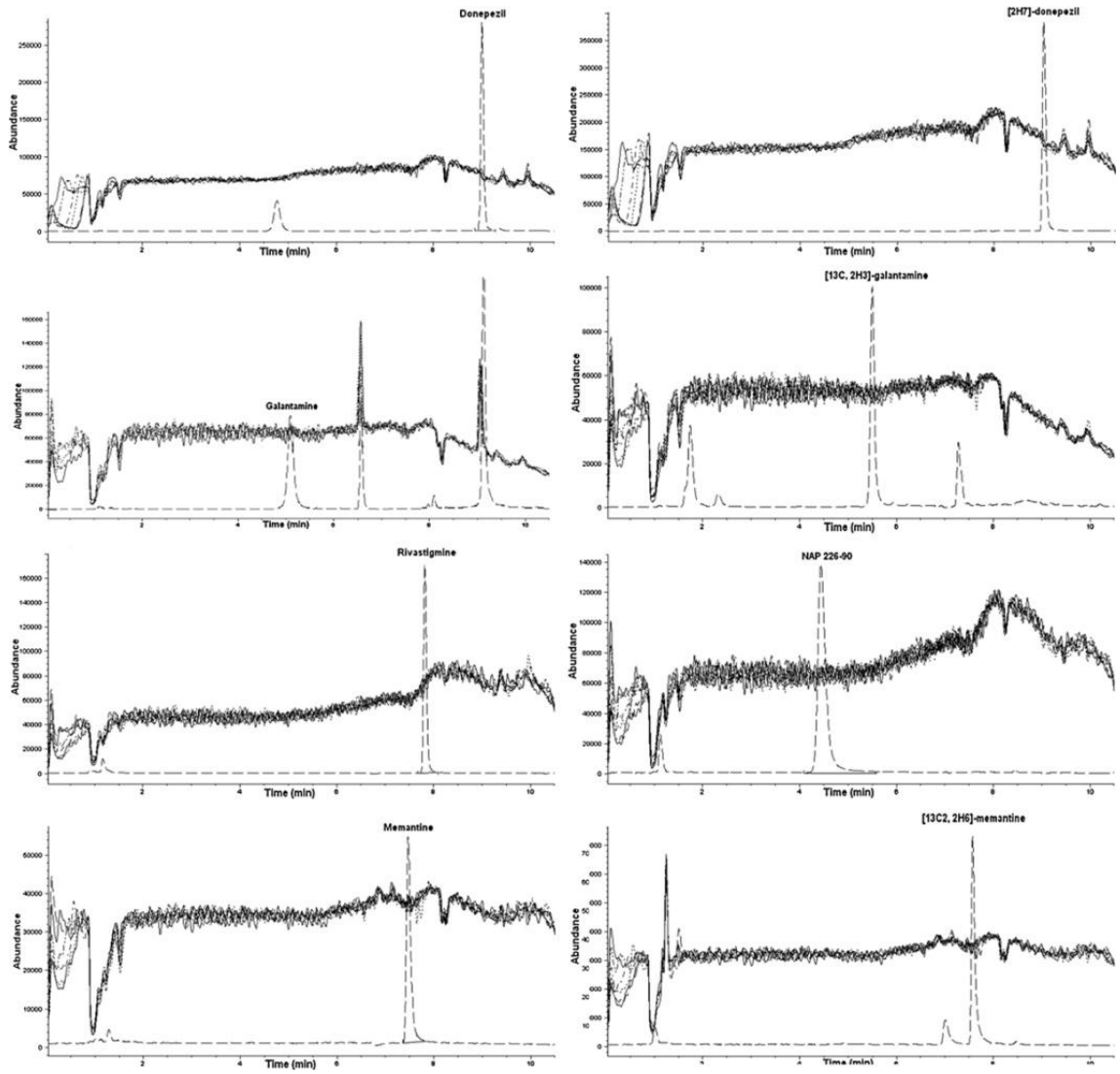

Figure 3: Chromatogram of 6 blank plasma extracts with post-column infusion of the analytes.

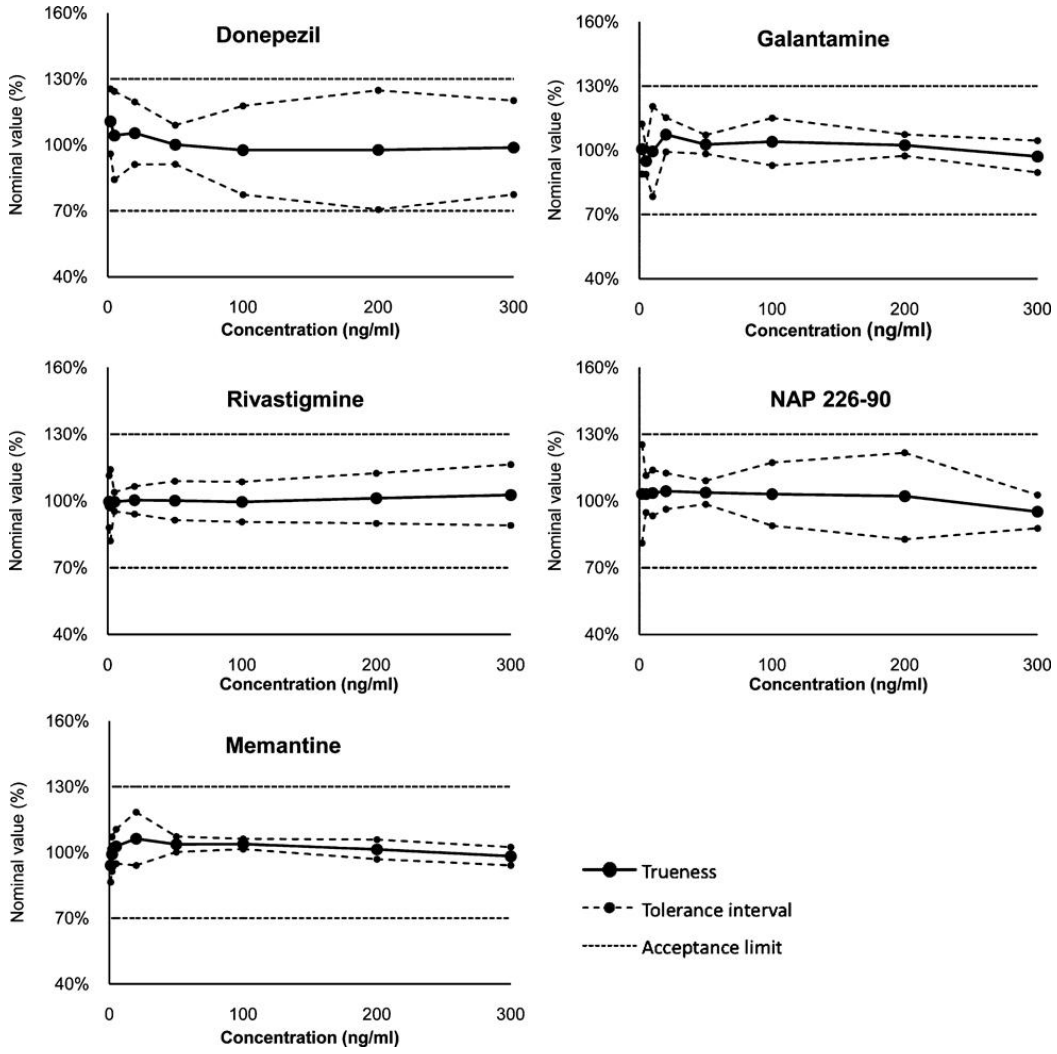

Figure 4: Accuracy profiles within the acceptance limit ($\lambda = \pm 30\%$), and with upper and lower β-expectation tolerance intervals ($\beta = 90\%$) for each compound in the dosing range.

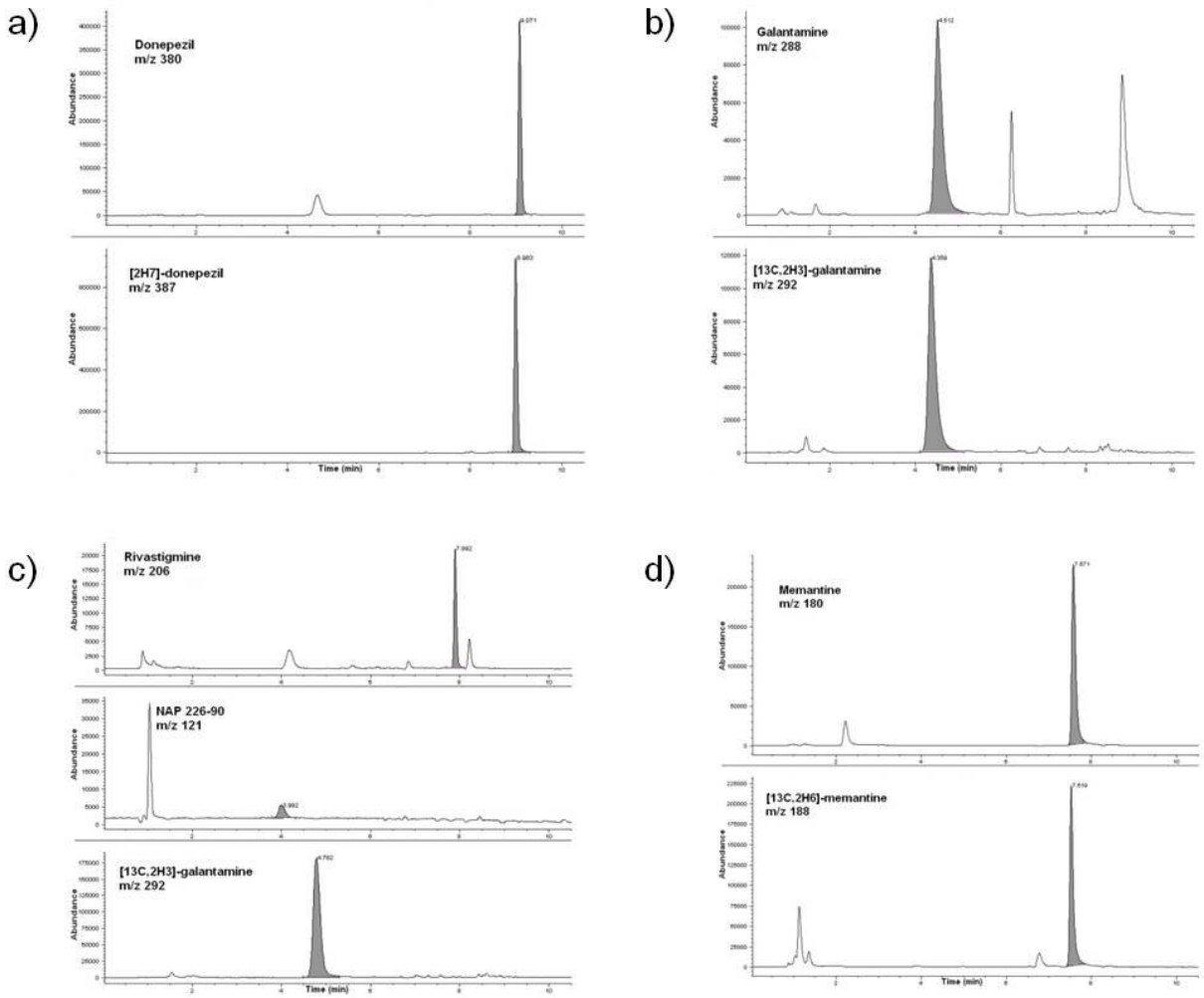

Figure 5: Representative chromatograms of patients receiving different antidementia drugs a) donepezil (60 ng/ml), b) galantamine (73 ng/ml), c) rivastigmine (5 ng/ml, NAP 226-90 3 ng/ml) and d) memantine (108 ng/ml).

Table 1: Indivual MS settings and typical retention times of the analytes.

	$[M+H]^+$ (m/z)	Fragment ion (m/z)	Fragmentor Voltage (V)	t_R (min)[b]
Donepezil	380.1[a]		150	9.0
Rivastigmine	251.1	206.1[a]	160	7.8
NAP 226-90	166.1	121.1[a]	140	3.7
Galantamine	288.1[a]		130	4.8
Memantine	180.1[a]	163.1	100	7.4
$[^2H_7]$-donepezil	387.2[a]		150	8.9
$[^{13}C,^2H_3]$-galantamine	292.1[a]		130	4.7
$[^{13}C_2,^2H_6]$-memantine	188.2[a]	171.2	100	7.4

a) Used for quantification of the compound
b) t_R: Retention time

Table 2: Matrix effects (ME), extraction recovery (RE), process efficiency (PE)

	Nominal conc. (n=6)	ME % (%CV)	RE % (%CV)	PE% (%CV)
Donepezil	10	104 (2)	95 (3)	98 (2)
	150	114 (5)	97 (6)	111 (2)
Rivastigmine	10	101 (1)	93 (1)	95 (1)
	150	107 (2)	90 (4)	97 (3)
NAP 226-90	10	104 (1)	96 (3)	99 (3)
	150	107 (2)	96 (3)	103 (2)
Galantamine	10	85 (2)	96 (1)	82 (2)
	300	106 (1)	93 (1)	98 (2)
Memantine	10	98 (2)	88 (2)	86 (3)
	300	103 (2)	93 (5)	96 (5)
$[^2H_7]$-donepezil	50	99 (2)	98 (3)	97 (3)
$[^{13}C,^2H_3]$-galantamine	50	106 (1)	94 (2)	100 (2)
$[^{13}C_2,^2H_6]$-memantine	50	101 (3)	93 (4)	94 (6)

Table 3: Trueness and precision of QC samples determined by repeated analysis in quadruplicates in three different series

	Nominal conc (ng/ml)	Trueness (%)	Precision (%)	
			Repeatability	Intermediate precision
Donepezil	2	110.7	2.7	5.5
	5	104.3	1.8	6.3
	20	105.3	4.4	6.3
	50	100.1	2.9	4.0
	100	97.6	1.5	6.2
	200	97.7	1.7	8.3
	300	98.8	4.9	8.5
	600/3	91.9	4.6	7.2
Rivastigmine	1	99.7	2.0	4.3
	2	98.0	2.0	5.3
	5	99.6	1.8	2.1
	20	100.4	2.7	3.1
	50	100.2	2.6	3.8
	100	99.6	2.1	3.6
	200	101.2	3.3	4.9
	300	102.7	4.9	6.4
	600/3	92.6	6.2	7.2
NAP 226-90	2	103.2	3.7	8.0
	4	103.1	3.0	3.9
	10	103.6	2.1	3.9
	20	104.4	3.8	4.1
	50	103.8	2.8	2.7
	100	103.1	1.6	4.6
	200	102.2	2.4	6.5
	300	95.2	2.6	3.4
	600/3	93.6	4.9	5.3
Galantamine	2	92.0	3.1	6.4
	4	92.1	3.4	3.1
	10	100.4	2.9	7.2
	20	109.7	4.1	4.7
	50	105.7	1.9	2.6
	100	107.2	1.7	4.6
	200	105.7	2.5	3.1
	300	100.2	2.3	3.1
	600/3	98.4	5	4.4
Memantine	1	94.1	4.0	4.1
	2	99.3	2.9	3.7
	5	102.8	3.1	3.7
	20	106.3	3.3	5.1
	50	103.8	1.8	1.8
	100	103.9	1.1	1.3
	200	101.4	2.4	2.1
	300	98.4	2.1	2.1
	600/3	94.0	4.7	5.3

Table 4: Plasma, post-preparative and whole blood stability expressed as percentage of the initial concentration (CV%)

Plasma	**Donepezil**		**Rivastigmine**[b]		**NAP 226-90**[b]		**Galantamine**		**Memantine**	
Nominal conc [ng/ml]	4 n=5	250 n=5	2 n=5	250 n=5	4 n=5	250 n=5	4 n=5	250 n=5	2 n=5	250 n=5
RT, 24 h	105 (3)	101 (3)	97 (2)	104 (2)	99 (3)	101 (3)	94 (6)	102 (3)	100 (4)	105 (2)
RT, 72 h	99 (1)	99 (3)	100 (3)	107 (2)	97 (3)	103 (2)	101 (4)	100 (2)	98 (3)	101 (2)
4°C, 72 h	-97 (2)	101 (2)	101 (2)	107 (3)	101 (2)	103 (3)	102 (3)	101 (2)	99 (4)	101 (2)
-20 °C, 3 months	95 (4)	102 (2)	109 (4)	97 (2)	96 (4)	99 (1)	99 (3)	98 (1)	101 (3)	98 (1)
Freeze/thaw cycle 1	97 (2)	99 (2)	99 (2)	112 (5)	95 (5)	103 (4)	102 (2)	104 (4)	103 (8)	104 (2)
Freeze/thaw cycle 2	97 (2)	95 (2)	104 (3)	113 (4)	103 (3)	103 (3)	104 (4)	102 (3)	108 (5)	104 (2)
Freeze/thaw cycle 3	95 (1)	94 (2)	104 (4)	114 (2)	101 (4)	105 (1)	103 (4)	102 (2)	103 (3)	103 (2)
Post-preparative, RT, 24 h	99 (5)	103 (2)	96 (2)	104 (3)	98 (4)	101 (1)	101 (7)	102 (3)	100 (1)	105 (1)
Post-preparative, 4°C, 48 h	96 (1)	101 (2)	98 (2)	98 (2)	101 (6)	101 (3)	99 (3)	101 (2)	107 (1)	99 (2)

Whole blood	**Donepezil**	**Rivastigmine**[b]		**NAP 226-90**[b]		**Galantamine**	**Memantine**
Nominal conc [ng/ml]	100 n=3	10 n=5	250 n=5	10 n=5	250 n=5	100 n=3	100 n=6
RT, 8 h	96 (5)	95 (4)	96 (4)	93 (3)	85 (5)	100 (1)	105 (5)
RT, 24 h	97 (4)	93 (4)	98 (5)	89 (7)	77 (5)	102 (1)	109 (3)
RT, 48 h	106 (4)	96 (3)	98 (4)	82 (7)	68 (9)	109 (2)	117 (3)
RT, 72 h	109 (4)	96 (3)	102 (3)	60 (11)	62 (12)	107 (1)	120)

a) RT: Room temperature
b) with addition of physostigmine

Table 5: Examples of plasma levels measured in three patients per drug dosage.

	Calibrated range (ng/ml)	Therapeutic range (ng/ml)	Plasma level Median (range) (ng/ml)	Daily dose (mg/d)
Donepezil	2 - 300	30 – 75[3]	27 (19 – 29)	5
			58 (34 - 59)	10
Rivastigmine	1 - 300	unknown	5 (5 – 14)	9.5 (patch)
NAP 226-90			5 (3 – 12)	
Rivastigmine			5 (1 – 22)	9 (capsule)
NAP 226-90			3 (2 – 7)	
Galantamine	2 - 300	30 – 100[3]	44 (38 – 84)	16
			73 (35 – 103)	24
Memantine	1 - 300	Unknown[9]	63 (15 – 75)	10
			146 (82 – 163)	20

3.5 Article III: Simultaneous determination of antidementia drugs in human plasma: Procedure transfer from HPLC-MS to UPLC-MS/MS

Summary

In this article, the transfer of the HPLC-MS method for the simultaneous determination of antidementia drugs to UPLC-MS/MS is described. The UPLC-MS/MS procedure allows to reduce the required amount of plasma, to use a simplified sample preparation, and to obtain a higher sensitivity and specificity with a much shortened run-time. The UPLC-MS/MS method was successfully validated. Moreover, a method comparison was performed with patients' samples, showing similar results between the HPLC-MS and UPLC-MS/MS procedures.

Plasma concentration measurements of antidementia drugs have recently been added to the accredited analyses proposed by our TDM service. Because of its numerous advantages, the analyses are performed by the UPLC-MS/MS procedure reported in this article. However, the HPLC-MS method can be used as a backup method.

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SIMULTANEOUS DETERMINATION OF ANTIDEMENTIA DRUGS IN HUMAN PLASMA: PROCEDURE TRANSFER FROM HPLC-MS TO UPLC-MS/MS

Muriel Noetzli[1], Nicolas Ansermot[1], Maria Dobrinas[1], Chin B Eap[1,2]

1. Unit of Biochemistry and Clinical Psychopharmacology, Centre for Psychiatric Neuroscience, Department of Psychiatry, CHUV, University of Lausanne, Hospital of Cery, Prilly, Switzerland.
2. School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Boulevard d'Yvoy 20, 1211 Geneva 4, Switzerland.

Author contributions

Concept: C.B. Eap

Method development: M. Noetzli

Scientific inputs for the method development: all authors

Drafting of the manuscript: M. Noetzli

Critical revision of the manuscript for important intellectual content: all authors

Abstract

A previously developed high performance liquid chromatography mass spectrometry (HPLC-MS) procedure for the simultaneous determination of antidementia drugs, including donepezil, galantamine, memantine, rivastigmine and its metabolite NAP 226-90, was transferred to an ultra performance liquid chromatography system coupled to a tandem mass spectrometer (UPLC-MS/MS). The drugs and their internal standards ([$^{2}H_{7}$]-donepezil, [^{13}C,$^{2}H_{3}$]-galantamine, [$^{13}C_{2}$,$^{2}H_{6}$]-memantine, [$^{2}H_{6}$]-rivastigmine) were extracted from 250 µL human plasma by protein precipitation with acetonitrile. Chromatographic separation was achieved on a reverse phase column (BEH C18 2.1x50mm; 1.7µm) with a gradient elution of an ammonium acetate buffer at pH 9.3 and acetonitrile at a flow rate of 0.4 mL/min and an overall run time of 4.5 min. The analytes were detected on a tandem quadrupole mass spectrometer operated in positive electrospray ionization mode, and quantification was performed using multiple reaction monitoring. The method was validated according to the recommendations of international guidelines over a calibration range of 1-300 ng/mL for donepezil, galantamine and memantine, and 0.2-50 ng/mL for rivastimgine and NAP 226-90. The trueness (86-108%), repeatability (0.8-8.3%), intermediate precision (2.3-10.9%) and selectivity of the method were found to be satisfactory. Matrix effects variability was inferior to 15% for the analytes and inferior to 5% after correction by internal standards. A method comparison was performed with patients' samples showing similar results between the HPLC-MS and UPLC-MS/MS procedures. Thus, this validated UPLC-MS/MS method allows to reduce the required amount of plasma, to use a simplified sample preparation, and to obtain a higher sensitivity and specificity with a much shortened run-time.

1. Introduction

Four drugs are currently used for the symptomatic treatment of dementia, the acetylcholinesterase inhibitors donepezil, galantamine and rivastigmine, and the N-methyl-D-aspartate (NMDA) receptor antagonist memantine. The chemical structures of the four antidementia drugs and the major metabolite of rivastigmine, NAP 226-90, are presented in Figure 1.

Therapeutic drug monitoring (TDM) is a well known tool for optimization of pharmacotherapy. By maintaining patients' drug plasma concentrations in the target range through individual dose adaption, efficacy and safety of many treatments, including psychotropic drugs, can be improved.[1,2] Even though little evidence exists, several factors indicate that TDM might also be beneficial for antidementia drugs.[1] A high inter-individual variability in response to treatment has been shown,[3-5] which might partly be due to the high inter-individual variabilites in plasma concentrations.[6-8] In elderly people, the presence of comorbidities and multiple comedication leading to drug-drug interactions, as well as genetic variations in metabolizing enzymes and transporters, might be causes of the observed inter-individual variabilities in plasma concentrations. Moreover, non-adherence to the treatment could be revealed by TDM, which is a particular problem in patients with cognitive deficits.[9]

We previously published a high performance liquid chromatography mass spectrometry (HPLC-MS) procedure for the simultaneous determination of the four antidementia drugs in human plasma for TDM.[10] To our knowledge, no other analytical methods allowing the simultaneous quantification of all four drugs have been published. However, several HPLC-MS/MS methods are described quantifying single compounds, sometimes with their metabolites, in human plasma.[11-21] Sample preparation was performed using solid phase extraction (SPE), liquid-liquid extraction (LLE) or protein precipitation.

In the present study, we aimed to transfer the previously developed HPLC-MS method to an ultra performance liquid chromatography system coupled to a tandem mass spectrometer (UPLC-MS/MS) to analyze the compounds by the most sensitive and specific methodology today available with a minimized run time per sample and a simplified extraction procedure of the drugs from plasma. UPLC technology has demonstrated significant advantages with respect to speed, sensitivity and resolution,[22] and detection by tandem MS further increases the sensitivity and specificity of the method. The UPLC-MS/MS procedure was fully validated and its performance evaluated by comparing the results of patients' plasma concentration measurements obtained by UPLC-MS/MS with the results previously obtained by HPLC-MS. The UPLC-MS/MS procedure is presently used in our laboratory for TDM in patients receiving antidementia drugs.

2. Experimental

2.1 Chemicals and Reagents

The drugs were kindly provided by their manufacturers: donepezil HCl by Eisai Co., Ltd (Tokyo, Japan), galantamine HBr by Janssen-Cilag (Beerse, Belgium), memantine HCl by Merz (Frankfurt/Main, Germany), rivastigmine hydrogen tartrate and its metabolite NAP 226-90 by Novartis (Basel, Switzerland). The internal standards (IS) [$^{2}H_{7}$]-donepezil, [^{13}C,$^{2}H_{3}$]-galantamine HCl, [$^{13}C_{2}$,$^{2}H_{6}$]-memantine HCl and [$^{2}H_{6}$]-rivastigmine hydrogen tartrate were purchased from Alsachim (Strasbourg, France). Biosolv® UPLC-grade acetonitrile, ammonium acetate (puriss p.a. for mass spectrometry) and physostigmine hemisulfate (eserine) were bought from Sigma-Aldrich (Buchs, Switzerland). Ultrapure water was obtained from a Milli-Q® RG with a QPAQ2 column system (Millipore, Billerica, MA, USA). All chemicals were of analytical grade. For the preparation of calibration standards (CS) and quality control (QC) samples and the evaluation of matrix effects, more than 10 different batches of human plasma from outdated blood donation units were obtained from the hospital's blood transfusion center (CHUV, Lausanne, Switzerland).

2.2 Equipment

The liquid chromatography system consisted of a Waters Acquity UPLC instrument equipped with a binary pump and a 96-vial autosampler (Waters, Milford, MA, USA). Chromatographic separation was performed on a BEH C18 column (2.1 x 50 mm; 1.7 µm) (Waters) equipped with a BEH C18 cartridge (2.1 x 5 mm; 1.7 µm). Analyses were carried out in an air conditioned room at 22°C and the

autosampler was kept at 8°C. The chromatographic system was coupled to a tandem quadrupole MS (TQD) (Waters) equipped with an electrospray ionization interface operated in positive ionization mode (ESI+). Data acquisition handling and instrument control were performed by the Masslynx software version V4.1 (Waters).

2.3 Stock and Working Solutions

Stock solutions of the analytes were prepared at 1 mg/mL (as base) in methanol and stored at -20°C. By dilution of the stock solutions with 0.01 N HCl, working solutions at 100 µg/mL were obtained and likewise stored at -20°C. CS and QC samples were prepared independently by spiking blank plasma at different concentrations with freshly made dilutions of the working solution at 0.01, 0.1, 1 and 10 µg/mL in 0.01 N HCl. CS and QC samples were analyzed immediately or stored at -20°C until analysis. The stock solutions of the IS were prepared at 1 mg/mL in methanol and stored at -20°C. They were diluted with acetonitrile to give a single IS working solution at 0.25 µg/mL ([$^{2}H_{7}$]-donepezil, [^{13}C,$^{2}H_{3}$]-galantamine, [$^{13}C_{2}$,$^{2}H_{6}$]-memantine) and 0.075 µg/mL ([$^{2}H_{6}$]-rivastigmine), respectively. To inhibit the enzymatic *in-vitro* hydrolysis of rivastigmine to its metabolite NAP 226-90 by plasma esterases, 100 µL of a 0.001 M physostigmine solution was added to every 1 mL of plasma used for the preparation of CS and QC samples.[19]

2.4 Sample Preparation

Plasma samples (250 µL) were mixed with 50 µL of IS solution and 750 µL acetonitrile were added for protein precipitation. The samples were vortex-mixed, sonificated for 30 s, and centrifuged for 10 min at 16000 x *g* (12610 rpm) on an Eppendorf Centrifuge 5430 (Eppendorf AG, Hamburg, Germany). The supernatants (900 µL) were transferred into polypropylene tubes and evaporated to dryness (N_2 flow, 45°C). The solid residues were reconstituted in 100 µL of the mobile phase at initial conditions (buffer-acetonitrile 80:20, *v/v*), vortex-mixed and again centrifuged for 10 min at 16000 x *g*. Finally, the supernatants (90 µL) were transferred into glass vials prior to injection into the UPLC MS/MS system.

2.5 UPLC-MS/MS conditions

The stationary phase used in HPLC and UPLC method development was based on the same technology. Thus, the established HPLC gradient, using an ammonium acetate buffer (pH 9.3; 50 mM for HPLC and 20 mM for UPLC) (solution A) and acetonitrile (solution B) as mobile phase, was translated to UPLC conditions by means of the HPLC calculator tool of the University of Geneva, Switzerland.[23] The obtained UPLC conditions were further improved and a suitable separation was achieved at a flow rate of 0.4 mL/min using the following stepwise elution program with an overall run time of 4.5 min: 20% of B maintained for 1.7 min, gradient to 35% of B from 1.7 to 1.75 min, hold at 35% of B from 1.75 to 2.2 min, gradient to 80% of B from 2.2 to 2.9 min, hold at 80% of B from 2.9 min to 3.5 min. The gradient was followed by rinsing with 95% of B from 3.7 to 4.0 min and a reconditioning step at initial conditions from 4.2 to 4.5 min. Of each sample 5 µL were injected.

Detection was performed using three multiple reaction monitoring (MRM) functions with the following transitions (Table 1): function 1 *m/z* 166.0→121 (NAP 226-90), 288.1→213 (galantamine), 292.1→213 ([^{13}C,$^{2}H_3$]-galantamine); function 2 *m/z* 180.1→163 (memantine), 188.1→171 ([$^{13}C_2$,$^{2}H_6$]-memantine), 251.0→206 (rivastigmine), 257.0→206 ([$^{2}H_6$]-rivastigmine); function 3 *m/z* 380.2→91 (donepezil), 387.2→98 ([$^{2}H_7$]-donepezil). For each function the dwell times were automatically assigned (Table 1). Nitrogen was used as desolvation gas at a flow rate of 800 L/h and a temperature of 400°C, and argon as collision gas at a flow rate of 0.35 mL/min. Source temperature was set at 150°C and capillary voltage at 3 kV. The cone voltage and the collision energy were optimized for all of the compounds separately by direct infusion of a solution at 1 µg/mL in 0.01 N HCl into the MS/MS at a flow rate of 10 µL/min and in combined mode with the mobile phase (60% solution A/40% solution B). The cone voltage was tested in MS scan mode (values from 10 to 60 eV) and the collision energy in product scan mode (values from 5 to 50 eV). The settings producing the highest signal intensities of parent and product ions were retained (Table 1).

2.6 Method Validation

The method validation was based on the recommendations of the "Société Française des Sciences et Techniques Pharmaceutiques" and on the two guidelines for bioanalytical method validation published online by the US Food and Drug Administration and by the European Medicines Agency.[24-26]

2.6.1 Selectivity, carry-over and psychiatric comedication

Method selectivity was ascertained by analyzing plasma extracts from 10 batches of blank plasma for interfering peaks at the retention time of the analytes and IS. Moreover, carry-over effects were investigated by determining the peak area of the compounds in blank plasma injected after spiked samples at three different concentrations over the calibration range (donepezil, galantamine, memantine at 75, 150 and 300 ng/mL; rivastigmine, NAP 226-90 at 12, 24 and 50 ng/mL).

Additionally, blank plasma was spiked with the following psychiatric drugs and some of their metabolites, and analyzed by the same procedure to investigate the influence of potential comedication: amitriptyline, amisulpride, aripiprazole, atomoxetine, dehydro-aripiprazole, bupropion, 3-hydroxy-bupropion, caffeine, chlorpromazine, citalopram, desmethyl-citalopram, clomipramine, desmethyl-clomipramine, clopenthixol, clozapine, N-oxid-clozapine, norclozapine, desipramine, duloxetine, fluoxetine, norfluoxetine, flupenthixol, fluvoxamine, haloperidol, imipramine, loxapine, maprotiline, mianserin, desmethyl-mianserin, midazolam, 1-hydroxy-midazolam, mirtazapine, 8-hydroxy-desmethyl-mirtazapine, moclobemide, N-oxid-moclobemide, 3'-oxo-moclobemide, morphine, nicotine, nortriptyline, olanzapine, paroxetine, quetiapine, reboxetine, risperidone, 9-hydroxy-risperidone, sertindole, dehydro-sertindole, sertraline, desmethyl-sertraline, sulpiride, trimipramine, desmethyl-trimipramine, trazodone, venlafaxine, O-desmethyl-venlafaxine, N-desmethyl-venlafaxine, N-O-di-desmethyl-venlafaxine and ziprasidone. In the case of a similar retention time to the antidementia drugs, suppression of the signal was assessed by comparing the peak area of the analyte at 100 ng/mL (donepezil, galantamine, memantine) and 10 ng/mL (rivastigmine, NAP 226-90)

alone with the peak area of the analyte when injected with the potential interfering compound at a high therapeutic concentration.

2.6.2 Matrix Effects, Extraction Recovery and Process efficiency

Primarily, matrix effects were examined qualitatively by simultaneous post-column infusion of the analytes and IS into the MS/MS detector during chromatographic analyses of 6 different blank plasma extracts and mobile phase.[27,28] The compounds were infused at a concentration of 50 ng/mL (donepezil, galantamine, memantine), 10 ng/mL (rivastigmine, NAP 226-90) and 100 ng/mL (IS) with a flow rate of 10 µL/min, corresponding to the lower end concentration signal response of the analytes. Signal suppression or enhancement at the retention time of the analytes was investigated.

Subsequently, matrix effects, recoveries of extraction and process efficiencies were assessed quantitatively at low (donepezil, galantamine, memantine at 3 ng/mL; rivastigmine, NAP 226-90 at 0.6 ng/mL) and high (donepezil, galantamine, memantine at 250 ng/mL; rivastimine, NAP 226-90 at 40 ng/mL) concentration based on the approach of Matuszewski.[29] Three sets of samples were processed as follows:

(A): Pure standard solution samples of the analytes and IS in the reconstitution solvent (buffer-acetonitrile 20:80, *v/v*) injected directly onto the column.

(B): Duplicates of plasma extract samples from 6 different sources spiked with the analytes and IS after extraction.

(C): Duplicates of plasma samples from 6 different sources (same as B) spiked with the analytes and IS before extraction.

For calculations, the mean peak area of the duplicates was used. The matrix effect (ME) was evaluated for each analyte and IS by calculating the ratio of the peak area in the presence of the matrix (samples spiked after extraction) to the peak area in absence of the matrix (pure standard) and expressed in percentage (ME = B/A). The recovery of extraction (RE) was determined by comparing the peak area of the pre-extraction spiked (C) to the post-extraction spiked samples (B) (RE = C/B). The overall process efficiency (PE), taking into account ME and RE, was assessed by calculating the ratio of the peak area of the pre-extraction spiked samples to the peak area of the pure standard (PE = C/A). Of all three parameters, the variability between the different plasma batches was evaluated and expressed as coefficient of variation (CV%). A value ≤15% was considered satisfactory. The same parameters and respective CVs were calculated considering the IS-normalized peak areas for each analyte.

2.6.3 Trueness and Precision

Three validation series were performed on independent days to determine the trueness and precision of the method. Duplicates of CS and quadruplicates of QC samples were set at 8 different levels covering the expected range of concentrations in patients:[8,30-32] 1, 2, 5, 20, 50, 100, 200, 300 ng/mL

for donepezil, galantamine and memantine, and 0.2, 0.5, 1, 2, 5, 10, 20, 50 ng/mL for rivastigmine and NAP 226-90. Results were based on the peak area ratio of the analytes and their IS. [$^{2}H_{7}$]-donepezil, [^{13}C,$^{2}H_{3}$]-galantamine, [$^{2}H_{6}$]-rivastigmine and [$^{13}C_{2}$,$^{2}H_{6}$]-memantine were used as IS for their respective analyte, whereas [$^{2}H_{6}$]-rivastigmine was additionally used for the metabolite of rivastigmine NAP 226-90. The QC samples were analyzed against the calibration curve of the same run and the trueness of each concentration level was expressed as percentage of the theoretical value. Precision was estimated by means of repeatability (intra-day variance) and intermediate precision (sum of intra-day and inter-day variances) and expressed as coefficients of variation (CV%).[33] Accuracy profiles within the acceptance limits (λ=±30%) and with β-expectation tolerance intervals (β=90%) were established for each compound.[33,34] Moreover, the linearity of the method was assessed applying a regression model to the recalculated QC concentrations versus theoretical concentrations.

In each validation run, four QC samples at 600 ng/mL (donepezil, galantamine, memantine) and 100 ng/mL (rivastigmine, NAP 226-90) were included to assess dilution integrity in case of a patient's plasma concentration exceeding the highest CS. The trueness and precision of these samples were determined carrying out a two-fold dilution with blank plasma prior to extraction.

2.6.4 Stability

The stability of all compounds in plasma and whole blood was assessed previously using the HPLC-MS procedure.[10] The *in vitro* degradation of rivastigmine was stopped by addition of the esterase inhibitor physostigmine to the samples. In the present study, additional tests were performed to investigate the stability of rivastigmine and NAP 226-90 in whole blood and plasma collected in commercially available blood sampling tubes containing sodium fluoride (1 mg NaF, 1.2 mg K-EDTA per mL), another esterase inhibitor.[35] Whole blood and plasma of 5 different persons were spiked with rivastigmine and NAP 226-90 at low (2 ng/mL) and high (20 ng/mL) concentration and different sets of aliquots were prepared. The stability was assessed after storage at ambient temperature for 24 h, 48 h and 72 h. In addition, a set of plasma aliquots was stored for 2 weeks at -20°C to investigate a longer storage in the freezer before analysis. The stability was evaluated by calculating the percentage of the initial concentration in the different aliquots.

Furthermore, the post-preparative stability was assessed for all compounds by leaving the processed samples up to 48 h on the autosampler at 8°C before reanalysis.

2.7 Method comparison between HPLC-MS and UPLC-MS/MS

Several patients' samples (33 for galantamine, 40 for donepezil, memantine and rivastigmine), previously quantified by HPLC-MS, were reanalyzed in different series by the described UPLC-MS/MS procedure. For NAP 226-90, instability was observed in patients' samples after storage for more than one year and multiple thaw/freeze cycles, therefore, aliquots of 28 spiked plasma samples with concentrations covering the dosage range were analyzed with both procedures to perform the comparison. For all analytes, the correlation between the two methods was tested by a Passing-

Bablok fit[36,37] and the mean bias was assessed by Bland-Altman plots[38] (Analyze-it, Microsoft Excel 2007).

3. Results and Discussion

3.1 Sample preparation

In the original HPLC-MS method, the drugs were isolated from 500 µL plasma by SPE, which is a powerful procedure to obtain clean extracts. However, the higher specificity of the UPLC-MS/MS compared to HPLC-MS allows the analysis of less clean extracts with satisfactory results. Therefore, the extraction procedure was simplified and a protein precipitation with acetonitrile was used with the advantages of a faster sample preparation and lower costs. In addition, the amount of required plasma was reduced to 250 µL. The extraction recoveries were comprised between 77% and 96%, with the exception of memantine and [$^{13}C_2$,$^{2}H_6$]-memantine for which the recoveries were between 46% and 54% (Table 2). Moreover, the repeatability was good for all compounds with CVs below 11% for the analytes and IS alone, and below 6% when the analyte/IS ratios were considered.

3.2 Transfer of Chromatographic Conditions and Optimization of MS/MS Conditions

The chromatographic conditions of the HPLC method with an overall run time of 15 min were translated to the UPLC system using the HPLC calculator tool from the University of Geneva.[23] The gradient was then optimized to achieve a satisfactory separation of the compounds. Compared to the original method, the concentration of the ammonium acetate buffer at pH 9.3 was reduced from 50 mM to 20 mM and instead of an X-Bridge column (2.1x100 mm; 3.5 µm), a BEH C18 column (2.1 x 50 mm; 1.7 µm) was used, which is based on the same stationary phase technology but packed with sub-2 µm particles. Elution was realized at a flow rate of 0.4 mL/min and the overall run time was 4.5 min. The retention times of the analytes are listed in Table 1 and a chromatographic profile of a QC plasma sample at the lower limit of quantification (LLOQ) is shown in Figure 2.

To improve the robustness of an analytical method, it is highly recommended to use isotope-labeled IS for quantification.[39,40] They compensate for signal alterations due to matrix effects and for variability in the extraction procedure. In the described method, isotope-labeled IS were used for donepezil, galantamine, rivastigmine and memantine, co-eluting with their respective analyte. For NAP 226-90, [$^{2}H_6$]-rivastigmine was used as IS after verification of the absence of signal suppression of rivastigmine on [$^{2}H_6$]-rivastigmine at the highest calibration level.

To find optimal ionization conditions, different settings of the cone voltage and of the collision energy were evaluated for each analyte. Satisfactory results were obtained with cone voltage values between 20 and 45 eV and collision energy values between 15 and 35 eV (Table 1). The detection was performed in MRM mode using three different functions, each of which monitored one or two analytes with their respective IS. Compared to the method using a unique MRM function, the variability of replicate injections was improved and the sensitivity was increased due to the higher dwell times.

3.3 Validation

3.3.1 Selectivity, Carryover and Comedication with Psychoactive Drugs

No peaks from endogenous compounds were observed at the analytes' retention times in any of the 10 blank plasma extracts evaluated. Moreover, no significant cross talk was observed between the isotope-labeled IS and the parent compounds. Injection of blank plasma after three different concentrations over the calibration range revealed no significant carry-over effects for all substances with the exception of donepezil. Even though different needle washes were investigated, a carry-over of 0.15% persisted for this analyte. The peak area of the carry-over should quantitatively represent less than 20% of the peak area of the analyte at LLOQ.[26] To meet this criterion, a blank sample has to be injected after the highest CS and between two subsequent injections of donepezil patients' samples.

Patients taking antidementia drugs frequently receive multiple comedication due to several comorbidities. Since comedication may potentially lead to analytical interferences, the influence of psychoactive drugs on the determination of antidementia drugs was assessed. For this purpose, plasma samples spiked with several antidepressant and antipsychotic drugs, and some of their metabolites, were analyzed with the same procedure. The retention times of the psychoactive drugs were recorded. Six substances coeluted with the compounds of interest, namely donepezil with bupropion, clopenthixol and dehydro-sertindole, rivastigmine with desmethyl-citalopram and 1-hydroxy-midazolam and NAP 226-90 with nicotine. All coeluting substances were distinguished by MS/MS detection and no significant signal suppression was observed for the antidementia drugs when injected with these compounds. However, the use of comedication in elderly patients is not restrained to psychoactive drugs, thus, special attention has to be paid to this issue during routine use of the method. Nevertheless, by the use of the highly specific UPLC-MS/MS technology and of isotope-labeled IS compensating for potential signal suppression, the risk of analytical inferences with comedication has been minimized.

3.3.2 Matrix Effects

The detection by MS in ESI mode is known to be sensitive to matrix effects, which refers to signal enhancement or suppression by endogenous compounds present in the biological matrix.[22,28] Matrix effects were qualitatively studied by the means of direct infusion of the analytes and IS into the MS/MS detector during analysis of six different blank plasma extracts. At the retention time of the analytes, signal suppression was observed for donepezil and [$^{2}H_{7}$]-donepezil, and signal enhancement for memantine and [$^{13}C_{2}$,$^{2}H_{6}$]-memantine, whereas no interferences were detected for the other compounds (data not shown). Additionally, matrix effects were assessed quantitatively by comparing the peak area of the analytes and IS in the pure standard solution and in six different plasma batches spiked at low (3 times LLOQ) and high (80% upper limit of quantification (ULOQ)) concentration. The findings of the post-column infusion experiment were confirmed by the quantitative assessment, which revealed matrix effects of 79% and 81% for donepezil and [$^{2}H_{7}$]-donepezil, and 110% and 105% for memantine and [$^{13}C_{2}$,$^{2}H_{6}$]-memantine, respectively, at low concentration (Table 2). However, when the

ratios analyte/IS were used for calculation, the matrix effects were considerably smaller for donepezil and memantine (98%-105%), showing the compensating effect of the isotope-labeled IS. For the other compounds, the matrix effects were comprised between 92% and 103%, and between 95% and 102% when the analyte/IS ratios were considered (Table 2). Even more important than absolute matrix effects, is to have a low variability of these effects between the different plasma batches. This aim was achieved with CVs inferior to 15% for the compounds alone and inferior to 5% for the ratios analyte/IS (Table 2).

Finally, the process efficiencies, representing the combined effects of extraction recovery and matrix effects, were also found to be satisfactory with values ranging from 93% to 109% and CVs inferior to 7% for the analyte/IS ratios (Table 2).

3.3.3 Trueness and Precision

Three different validation series were performed on independent days. Eight CS were initially used for each compound covering the range from 1 to 300 ng/mL for donepezil, galantamine and memantine, and from 0.2 ng/mL to 50 ng/mL for rivastigmine and its metabolite NAP 226-90. Different calibration models were tested and the following four-point calibration curves were selected: 1, 20, 100 and 300 ng/mL for donepezil (quadratic regression model weighted 1/x), galantamine (linear regression model weighted $1/x^2$) and memantine (linear regression model weighted 1/x), and 0.2, 2, 20 and 50 ng/mL for rivastigmine and NAP 226-90 (linear regression model weighted 1/x in both cases).

The QC samples were analyzed against the calibration curve of the same run and the trueness, repeatability and intermediate precision at each concentration level were determined (Table 3). In accordance to the above-mentioned guidelines, the QC samples were within the specifications. The determined trueness met the acceptance criterion of 100±15% (LLOQ ±20%) with values comprised between 86% and 108%. Moreover, the values for repeatability and intermediate precision met the requirements of CVs ≤15% (LLOQ ≤20%) with values inferior to 8.3% and 10.9%, respectively. Consequently, the LLOQs were set at 1 ng/mL for donepezil, galantamine and memantine, and at 0.2 ng/mL for rivastigmine and NAP 226-90. The higher sensitivity of the UPLC-MS/MS technology compared to the HPLC-MS allowed to reduce the LLOQs of rivastigmine and NAP 226-90. Therefore, the calibration ranges were modified as follows to better correspond to the low concentrations observed in patients: from 1 to 300 ng/mL (rivastigmine) and 2 to 300 ng/mL (NAP 226-90) in HPLC-MS to 0.2 to 50 ng/mL (both analytes) in UPLC-MS/MS.

The accuracy profiles for each compound in the dosing range with β-expectation tolerance intervals (β=90%) are presented in Figure 3. All accuracy profiles are within the set acceptance limits of ±30% with the exception of NAP 226-90. A possible reason for the larger profile of NAP 226-90 might be the lack of a coeluting isotope-labeled IS which would compensate for variabilities in the extraction procedure and matrix effects. The larger accuracy profile of NAP 226-90 is probably not of clinical significance as NAP 226-90 is an inactive and non-toxic metabolite. However, NAP 226-90 was introduced in the method because it is an indicator of the extent of rivastigmine metabolism.

The dilution integrity was confirmed by a twofold dilution of QC samples at concentrations exceeding twofold the highest calibration level. The trueness and precision of the diluted samples met the acceptance criteria of 100±15% and CVs ≤15%, therefore, plasma sample of patients containing antidementia drugs at concentrations exceeding the ULOQ can be adequately diluted with blank plasma before analysis.

Furthermore, a linear regression model was applied to the recalculated QC concentrations versus theoretical concentrations to assess the linearity of the method. The following slopes 0.995, 0.973, 0.953, 1.005 and 0.867 and intercepts 0.200, 2.223, 0.332, 0.270 and 0.037 were obtained for donepezil, galantamine, memantine, rivastigmine and NAP 226-90, respectively. The corresponding determination coefficients were 0.997, 0.994, 0.953, 0.996 and 0.989, indicating that the developed method was linear for the tested compounds.

3.3.4 Stability

As previously described, the analytes were stable in plasma for 72 h at room temperature, up to 3 months at -20°C and after three freeze/thaw cycles. Moreover, the drugs were stable in K-EDTA whole blood for at least 24 h at room temperature, with the exception of NAP 226-90 for which a rapid degradation was observed.[10] Rivastigmine is metabolized to NAP 226-90 by esterases that are present in blood and plasma. To stop the *in-vitro* degradation of rivastigmine after blood sampling, the esterase inhibitor physostigmine was added to the samples. NaF is another known esterase inhibitor[35] and, in contrast to physostigmine, it is used in commercial blood sampling tubes to inhibit glucose degradation. For TDM of rivastigmine, using commercially available blood sampling tubes would be more convenient. Therefore, the stability of rivastigmine and NAP 226-90 in whole blood and plasma collected in NaF containing blood sampling tubes was tested over a period of 72 h at room temperature. The degradation of rivastigmine remained below 20% under the following conditions: up to 72 h in whole blood and 48 h in plasma at room temperature, and up to 2 weeks in plasma at -20°C (Table 4). The concentration of the metabolite NAP 226-70 increased proportionally to the decrease in rivastigmine concentration. Compared to physostigmine, the inhibition by NaF is less strong and thus the degradation of rivastigmine more rapid. For reasons of convenience, blood sampling tubes containing NaF could be used. However, to assure better precision of the measurement, the samples should be frozen as soon as possible.

Since a new extraction procedure was used, the post-preparative stability was retested with the UPLC-MS/MS method. All analytes were found to be stable in the injection solution up to 48 h at 8°C with values between 86% and 110% of the initial concentration (Table 4).

3.4 Method comparison between HPLC-MS and UPLC-MS/MS

A method comparison was performed by analyzing samples previously measured by HPLC-MS by the newly developed UPLC-MS/MS procedure. The Passing-Bablok regression equations and corresponding plots are presented in Figure 4. The 95% CI included the value 1 for the slope and the value zero for the intercept for donepezil, galantamine and memantine, indicating no statistically

significant difference between the methods. In contrast, the slope of rivastigmine and NAP 226-90 did not include the value 1, meaning that there is a proportional difference between the two methods. However, the mean bias obtained by the Altman-Bland plots were found to be small with 10.9% (95% CI 5.9% to 15.9%) and -5.6% (95% CI -7.3% to -3.9%) for rivastigmine and NAP 226-90, respectively. These differences may not be of clinical relevance and are possibly due to the time difference between the tests, the use of different stock solutions and the modified calibration ranges for rivastigmine and NAP 226-90.

4. Conclusion

The procedure developed on HPLC-MS for the determination of antidementia drugs in human plasma was successfully transferred to UPLC-MS/MS. Sample preparation was simplified by using protein precipitation instead of SPE and, due to the higher sensitivity of the tandem MS, the required amount of plasma was reduced from 500 µL to 250 µL. The calibration ranges of rivastigmine and NAP 226-90 were modified, with a decreased LLOQ of 0.2 ng/mL, to better correspond to plasma concentrations observed in patients. Moreover, the run time was shortened from 15 min to 4.5 min. The procedure was fully validated according to the recommendations of international guidelines. A method comparison between HPLC-MS and UPLC-MS/MS was performed showing similar results between the two procedures. Both methods are reliable and can be used for TDM in patients receiving antidementia drugs. However, the UPLC-MS/MS method is preferable with respect to specificity, sensitivity and speed and is presently used in the routine TDM service in our laboratory.

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Conflict of interest

The authors declare no conflict of interest.

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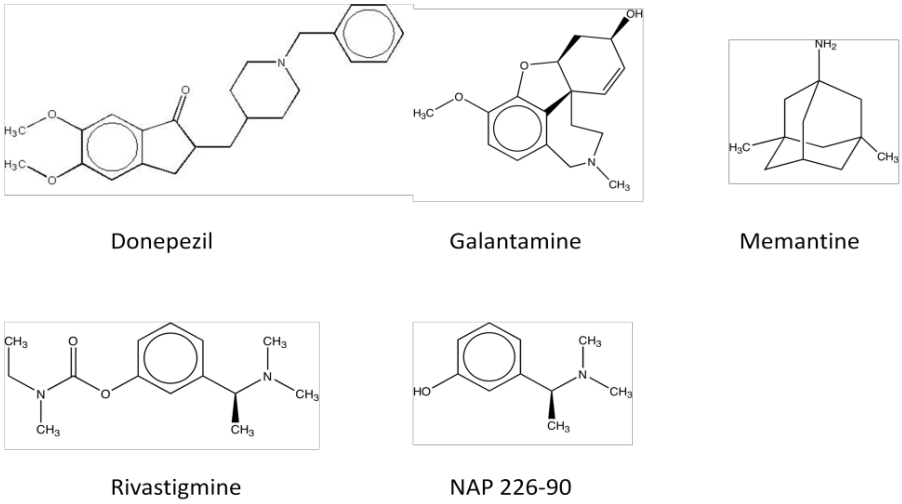

Figure 1: Chemical structures of the analytes.

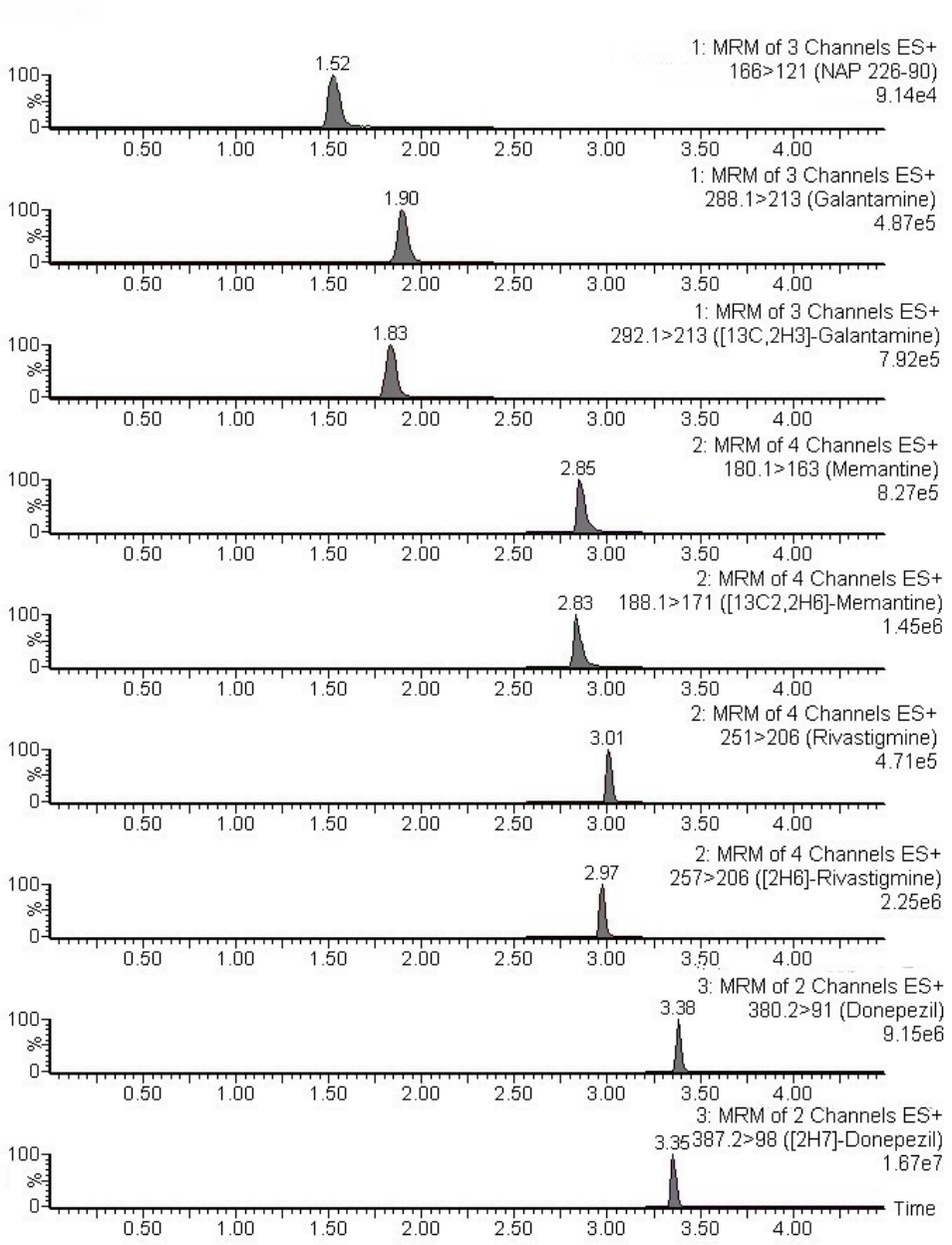

Figure 2: Chromatogram of a QC plasma sample at LLOQ (1 ng/mL donepezil, galantamine, memantine, 0.2 ng/mL rivastigmine, NAP 226-90, 50 ng/mL [$^{2}H_{7}$]-donepezil, [^{13}C,$^{2}H_{3}$]-galantamine, [$^{13}C_{2}$,$^{2}H_{6}$]-memantine and 15 ng/mL [$^{2}H_{6}$]-rivastigmine).

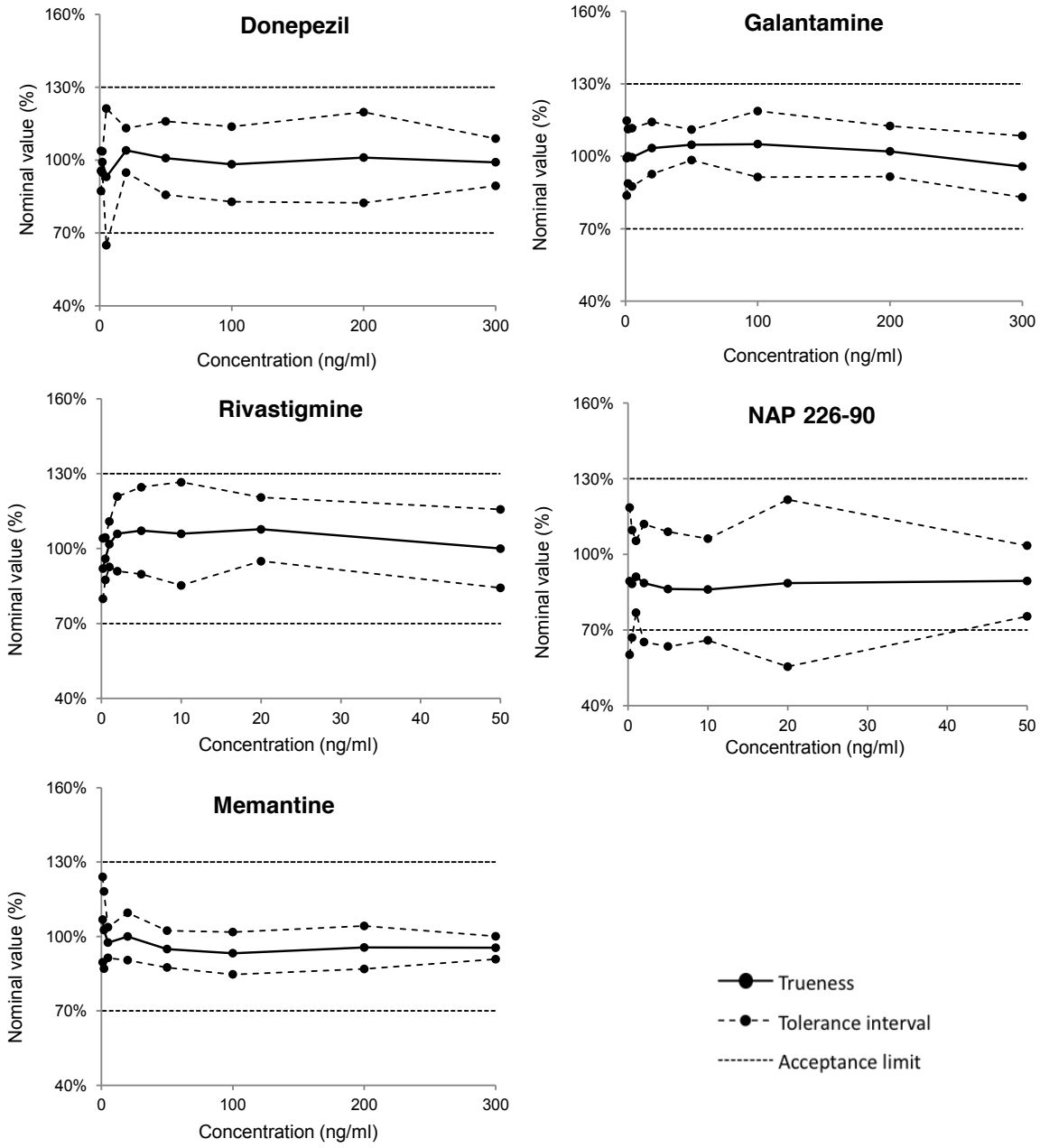

Figure 3: Accuracy profiles within the acceptance limit (λ=±30%) and with upper and lower β-expectation tolerance intervals (β=90%) for each compound in the dosing range.

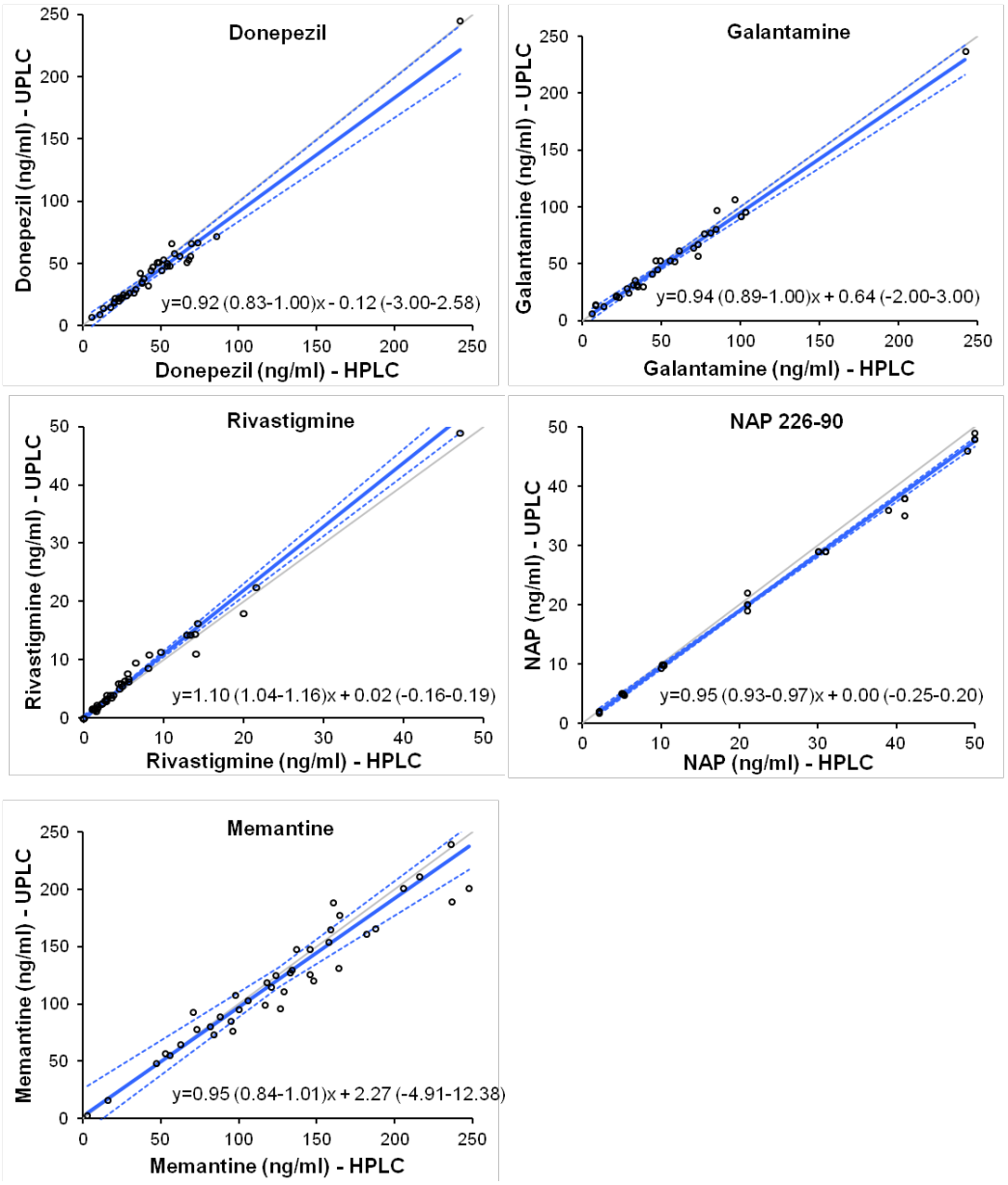

Figure 4: Passing-Bablok fits of the comparison between UPLC-MS/MS and HPLC-MS. In the plots the regression lines (solid line), the 95% confidence intervals for the regression line (dashed lines, values in brackets) and the identity lines (x=y, gray line) are shown.

Table 1: MRM parameters and retention times of the analytes and IS.

	Parent (m/z)	Fragment (m/z)	Cone voltage	Collision energy	Dwell time (ms)	t_R (min)[a]
Function 1 (0-2.4 min)						
NAP 226-90	166	121	20	15	171	1.5
Galantamine	288	213	30	25	171	1.9
[^{13}C,$^{2}H_3$]-galantamine	292	213	30	25	171	1.8
Function 2 (2.5-3.2 min)						
Memantine	180	163	30	15	128	2.9
[$^{13}C_2$,$^{2}H_6$]-memantine	188	171	30	15	128	2.8
Rivastigmine	251	206	20	15	128	3.0
[$^{2}H_6$]-rivastigmine	257	206	20	15	128	3.0
Function 3 (3.2-4.5 min)						
Donepezil	380	91	45	35	261	3.4
[$^{2}H_7$]-donepezil	387	98	45	35	261	3.4

[a] t_R: Retention time.

Table 2: Matrix effects (ME), recovery of extraction (RE) and process efficiency (PE)

	Concentration (ng/mL)		ME % (CV%[a]) n=6		RE % (CV%[a]) n=6		PE % (CV%[a]) n=6	
	Low	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**
Donepezil	3	250	79 (4)	90 (4)	95 (6)	79 (4)	75 (9)	72 (7)
[$^{2}H_{7}$]-donepezil	50	50	81 (7)	86 (7)	96 (7)	77 (5)	78 (11)	66 (10)
Galantamine	3	250	97 (2)	92 (2)	80 (1)	85 (2)	77 (2)	79 (1)
[^{13}C,$^{2}H_{3}$]-galantamine	50	50	95 (2)	98 (2)	83 (3)	84 (2)	79 (2)	82 (1)
Rivastigmine	0.6	40	102 (2)	102 (1)	80 (2)	80 (2)	82 (2)	81 (2)
[$^{2}H_{6}$]-rivastigmine	15	15	103 (1)	101 (1)	81 (1)	82 (1)	83 (2)	83 (2)
NAP 226-90	0.6	40	99 (1)	97 (1)	78 (3)	78 (1)	77 (3)	76 (1)
Memantine	3	250	110 (14)	104 (7)	46 (7)	53 (9)	51 (16)	55 (15)
[$^{13}C_{2}$,$^{2}H_{6}$]-memantine	50	50	105 (9)	100 (4)	47 (6)	54 (10)	49 (12)	54 (12)
Donepezil/[$^{2}H_{7}$]-donepezil	3	250	98 (4)	105 (3)	99 (3)	103 (1)	96 (3)	109 (3)
Galantamine/[^{13}C,$^{2}H_{3}$]-galantamine	3	250	102 (3)	95 (2)	96 (3)	102 (3)	98 (2)	106 (2)
Rivastigmine/[$^{2}H_{6}$]-rivastigmine	0.6	40	99 (2)	101 (2)	99 (3)	98 (2)	98 (3)	98 (2)
NAP 226-90/[$^{2}H_{6}$]-rivastigmine	0.6	40	96 (2)	96 (1)	96 (3)	95 (1)	93 (3)	93 (2)
Memantine/[$^{13}C_{2}$,$^{2}H_{6}$]-memantine	3	250	105 (4)	104 (3)	97 (5)	98 (3)	102 (6)	103 (4)

[a]CV%: Coefficient of variation.

Table 3: Trueness and precision of quality control samples in quadruplicates determined in three different validation series.

	Nominal Concentration (ng/mL)	Trueness (%)	Precision (%)	
			Repeatability	Intermediate precision
Donepezil	1	95.5	3.3	4.0
	2	99.2	2.3	2.3
	5	93.1	2.9	9.1
	20	104.0	2.8	4.1
	50	100.8	1.8	5.0
	100	98.3	0.8	4.7
	200	101.0	2.1	6.1
	300	99.1	2.1	3.8
Galantamine	1	99.2	5.5	7.2
	2	100.0	6.0	6.0
	5	99.6	3.3	5.1
	20	103.4	3.7	5.0
	50	104.7	3.0	3.3
	100	105.1	4.9	6.4
	200	102.0	3.2	4.6
	300	95.8	4.7	6.0
Rivastigmine	0.2	92.0	6.5	6.5
	0.5	96.0	4.5	4.5
	1	101.8	3.6	4.4
	2	105.9	3.1	5.7
	5	107.2	2.4	5.9
	10	105.9	4.1	7.9
	20	107.7	4.0	5.7
	50	100.0	2.4	5.5
NAP 226-90	0.2	89.0	4.6	6.4
	0.5	88.2	6.1	8.0
	1	91.2	5.1	5.7
	2	88.7	2.6	7.1
	5	86.4	1.8	6.7
	10	86.2	1.9	6.0
	20	88.7	4.7	10.9
	50	89.6	1.8	4.3
Memantine	1	106.8	4.1	6.9
	2	102.6	8.3	8.3
	5	97.5	3.3	3.3
	20	100.0	4.5	4.9
	50	94.9	2.1	3.2
	100	93.2	2.6	3.7
	200	95.5	3.8	4.3
	300	95.4	2.2	2.4

Table 4: Postpreparative stability of all compounds and stability of rivastigmine and NAP 226-90 in sodium fluoride containing blood sampling tubes expressed as the percentage of the initial concentration (coefficient of variation %).

	Rivastigmine (n=5)		**NAP 226-90** (n=5)		**Donepezil** (n=5)		**Galantamine** (n=5)		**Memantine** (n=5)	
	Low	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**	**Low**	**High**
Nominal Concentration (ng/mL)	0.6	40	0.6	40	3	250	3	250	3	250
Post-preparative stability										
8°C, 24 h	100 (0)	103 (3)	107 (14)	100 (5)	97 (3)	97 (3)	86 (8)	100 (3)	98 (6)	97 (7)
8°C, 48 h	100 (0)	100 (4)	104 (9)	101 (4)	99 (4)	100 (5)	90 (5)	110 (1)	99 (5)	100 (6)
	Low	**High**	**Low**	**High**						
Nominal Concentration (ng/mL)	2	20	2	20						
Whole blood (NaF)										
RT[a], 24 h	95 (10)	94 (8)	100 (13)	101 (8)						
RT[a], 48 h	90 (5)	101 (5)	106 (6)	99 (12)						
RT[a], 72 h	84 (8)	89 (12)	110 (5)	110 (19)						
Plasma (NaF)										
RT[a], 24 h	93 (5)	94 (4)	108 (7)	110 (16)						
RT[a], 48 h	82 (8)	85 (4)	113 (8)	115 (1)						
RT[a], 72 h	69 (9)	76 (7)	110 (8)	121 (11)						
-20°C, 2 weeks	91 (4)	87 (2)	84 (10)	98 (7)						

Chapter 4: Results

4.1 Article IV: Relationship of genetic variants in *CYP2D6*, *CYP3A*, *POR*, *NR1I2* and *ABCB1* genes with steady-state donepezil plasma concentrations

Summary

In this article, the results of the pharmacogenetic study in patients treated with donepezil are described. The large inter-individual variability in donepezil plasma concentrations, reported in previous studies, was confirmed. The relationship of genetic variations in *CYP2D6, CYP3A, POR, NR1I2* and *ABCB1*, and of other clinical factors, with donepezil steady-state plasma concentrations was investigated in 129 older patients treated with this drug. The *CYP2D6* genotype appeared to be the major genetic factor contributing to the variability in plasma concentrations. Thus, CYP2D6 homozygous extensive metabolizers demonstrated significantly lower plasma concentrations than heterozygous extensive metabolizers and poor metabolizers, and higher plasma concentrations than ultrarapid metabolizers. Moreover, significantly higher plasma concentrations were observed in *NR1I2* rs1523130T carriers, which might be due to a lower CYP3A expression in these patients. Finally, the *POR**28T allele was associated with lower donepezil concentrations in CYP2D6 extensive metabolizers, which is in line with the higher CYP3A activity described in carriers of this allele. These findings contribute to a better understanding of donepezil pharmacokinetics and might help, with further research, to improve clinical effectiveness and tolerance of the treatment.

Article in preparation

RELATIONSHIP OF GENETIC VARIANTS IN *CYP2D6, CYP3A, POR, NR1I2* AND *ABCB1* GENES WITH STEADY-STATE DONEPEZIL PLASMA CONCENTRATIONS

Muriel Noetzli[1], Karsten Ebbing[2], Stephan Eyer[3], Laurence Wilhelm[4], Agnès Michon[5], Valérie Thomazic[5], Ioana Stancu[2], Abdel-Messieh Alnawaqil[6], Christophe Bula[3], Serge Zumbach[6], Michel Gaillard[2], Panteleimon Giannakopoulos[7], Armin von Gunten[2], Chin B Eap[1,8]

1. Unit of Pharmacogenetics and Clinical Psychopharmacology, Centre for Psychiatric Neuroscience, Department of Psychiatry, Lausanne University Hospital, Hospital of Cery, Prilly, Switzerland.
2. Service of Old Age Psychiatry, Department of Psychiatry, University Hospital Lausanne, Prilly-Lausanne, Switzerland.
3. Geriatric Medicine and Geriatric Rehabilitation, Department of medicine, University Hospital Lausanne, Lausanne, Switzerland.
4. Fondation Nant, Consultation Aigle, Aigle, Switzerland.
5. Division of Geriatric Psychiatry, Department of Psychiatry, University Hospital Geneva, Geneva, Switzerland
6. Fribourg Mental Health Network, Department of Psychiatry, Cantonal Hospital Fribourg, Marsens, Switzerland.
7. Divisions of General Psychiatry, Department of Mental Health and Psychiatry, University Hospital Geneva, Geneva, Switzerland.
8. School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Boulevard d'Yvoy 20, 1211 Geneva 4, Switzerland.

Author contributions

Study concept and design: C.B. Eap

Genetic and statistical analyses: M. Noetzli

Data interpretation: M. Noetzli, C.B. Eap

Drafting of the manuscript: M. Noetzli

Patient recruitment and study supervision: all authors

Critical revision of the manuscript for important intellectual content: all authors

ABSTRACT

Background: A large interindividual variability in plasma concentrations was observed in patients treated with donepezil, the most frequently prescribed antidementia drug. Donepezil is mainly metabolized by the enzymes CYP2D6 and CYP3A, and it is a substrate of the P-glycoprotein. We aimed to study the relationship of genetic variants influencing the activity of these enzymes and transporter, and of other clinical factors, with donepezil steady state plasma concentrations.

Methods: In this naturalistic cross-sectional study, 129 older patients treated with a constant dose of donepezil were included. The patients were genotyped for polymorphisms in *CYP2D6, CYP3A, POR, NR1I2* and *ABCB1* genes, and donepezil steady state plasma concentrations were determined.

Results: The *CYP2D6* genotype appeared to be the major genetic factor contributing to the variability in plasma concentrations. Thus, significant differences were found between CYP2D6 homozygous extensive metabolizers and heterozygous extensive metabolizers, poor metabolizers and ultrarapid metabolizers with median dose-adjusted plasma concentrations of 4.7 versus 5.3 (p=0.037), 6.3 (p=0.005) and 2.6 (p=0.005) ng/ml·mg, respectively. Significantly higher plasma concentrations were observed in *NR1I2* rs1523130T carriers (p=0.005), potentially having a lower CYP3A expression. Moreover, the *POR*28* was associated with lower donepezil concentrations in CYP2D6 extensive metabolizers (p=0.018).

Conclusions: Genetic variations, primarily in *CYP2D6*, significantly influenced donepezil plasma concentrations and might therefore have an impact on the efficacy and safety of the treatment.

INTRODUCTION

Donepezil is an acetylcholinesterase inhibitor widely used in the symptomatic treatment of Alzheimer's disease (AD). The inhibition of the acetylcholinesterase increases the level of acetylcholine in the synaptic cleft and thus compensates the cholinergic deficit observed in the AD pathology. Cholinergic adverse events (AE) are common and include gastrointestinal problems, dizziness, insomnia, fatigue and muscle cramps.[1] Several studies reported a large variability in drug response to donepezil among AD patients,[2,3] which might partly be due to the observed interindividual differences in plasma concentrations of the drug.[4] On one hand, these differences could be triggered by genetic variations in enzymes and transporters implicated in drug metabolism and elimination. On the other hand, in elderly patients, the presence of multiple comorbidities and polypharmacy resulting in drug-drug interactions might lead to changes in plasma concentrations.

Donepezil is metabolized in the liver by the cytochromes P450 (CYP) 2D6 and 3A. 6-*O*-desmethyl-donepezil is the only active metabolite with a comparable potency in AChE inhibition as donepezil. It is, however, only present at around 20% of the concentration of the parent drug.[5] Coadministration of drugs inhibiting CYP2D6 and CYP3A has been found to increase plasma concentrations of donepezil, showing the importance of these two enzymes in donepezil metabolism.[6,7]

Phenotypically, four types of CYP2D6 metabolizers can be distinguished: poor (PM), intermediate (IM), extensive (EM) and ultrarapid metabolizers (UM). Underlying genetic variations defining the CYP2D6 phenotypes are well known.[8] One clinical trial reported a non significant influence of the *CYP2D6* genotype on donepezil plasma concentrations.[4] However, this study enrolled a small number of subjects (n=42) without any PM. Several studies also investigated the influence of genetic variations in *CYP2D6* on response to donepezil treatment. Although discrepant, results from these studies suggest that patients with *CYP2D6* genotypes associated with no or low activity (i.e. leading to higher plasa concentrations) may have better clinical outcomes.[9-12]

The predictive information of genetic variations in *CYP3A* on enzyme activity is more limited.[13] CYP3A stands for a group of isozymes, namely CYP3A4, CYP3A5 and CYP3A7, which have a large overlap in substrate specificity. A clinical trial investigated the association of different *CYP3A4/5* polymorphisms on plasma concentrations and therapeutic outcome of donepezil. The results suggest no major impact of genetic variants in *CYP3A4/5* on donepezil metabolism.[14] Furthermore, donepezil is a substrate of the P-glycoprotein (P-gp),[15] a membrane transporter implicated in drug absorption, distribution and excretion. Several polymorphisms in the *ABCB1* gene, coding for P-gp, have been shown to influence its activity.[16] In the above mentioned study, patients homozygous for the *ABCB1* 1236T/2677T/3435T haplotype showed a tendency toward lower plasma concentrations.[14]

Genetic variations in other genes than *CYP3A4/5/7* could influence CYP3A activity. The cytochrome P450 oxidoreductase (POR) is a protein that transfers electrons from NADPH to microsomal CYP enzymes and might, therefore, be a general limiting factor for drug metabolizing capacity.[17] Many polymorphisms in the *POR* have been described, some of them changing the activity of the enzyme *in vitro*. The results of an *in vivo* study suggest a 1.6-fold increase in CYP3A activity among homozygous carriers of the common *POR*28* allele.[17] Furthermore, the Pregnane X receptor (PXR) regulates the expression of detoxifying drug-metabolic enzymes and transporters, including CYP3A4 and P-gp.[18] PXR is a nuclear receptor encoded by the *NR1I2* gene and is activated by a variety of xenobiotics and endogenous ligands. *In vitro* studies revealed genetic variants in the regulatory region of *NR1I2* associated with inducible and constitutive CYP3A4 expression.[19,20]

In the present study, we investigated the effect of genetic variations in *CYP2D6*, *CYP3A4/5/7*, *POR*, *NR1I2* and *ABCB1* on steady state donepezil plasma concentrations in a group of 129 older patients treated with donepezil. The relationship between plasma concentrations and genotypes on recorded side effects was also investigated.

METHODS

Study design and participants

This cross-sectional study was conducted at four hospitals (Lausanne, Geneva, Marsens, Aigle) and in affiliated nursing homes. A total of 129 patients treated with donepezil for at least one month with a

stable dose were included. 89% of the participants were inpatients and 11% were outpatients. One blood sampling was performed to measure donepezil plasma concentration, to evaluate renal and hepatic function (standard clinical laboratory tests: ALT, ASP, AP, GGT, bilirubin, urea, creatinine), and to extract DNA for the genetic analyses. The presence of adverse events, reported by the patients, doctors and/or nurses, was registered. Additionally, concomitant diseases, comedication, consumption of grapefruit, alcohol and tobacco were recorded.
The study was approved by the local ethics committees (Lausanne University Hospital, Geneva University Hospital, Cantonal Ethic Committee Fribourg) and conducted according to the Good Clinical Practices. Written informed consent, including consent for genetic analysis, was obtained from all patients or their legal representative.

Plasma concentration determination and estimation of trough concentration

Blood samples were collected on average 14 hours after medication intake (range: 1-28 hours) in EDTA blood tubes. After centrifugation, the plasma was stored at -20°C until analysis. Donepezil plasma concentrations were determined by high performance liquid chromatography coupled with mass spectrometry, as previously described.[21]

Trough concentrations at 24 hours after drug intake were estimated by the means of the following equation: $C_{trough}=C_{measured}*e^{-((\ln 2/t1/2)*dt)}$. According to literature, $t_{1/2}$ was considered as 70 hours [22-25] and dt as the time between medication intake and blood sampling. The maximal plasma concentration is reached around four hours after drug intake.[23-25] In patients in the first two hours of the absorption phase (n=12), the measured plasma concentration was regarded as trough concentration. In patients in the second part of the absorption phase (n=9), the above-mentioned equation was used to calculate the trough concentration assuming that most of the drug substance was already absorbed. Since linear pharmacokinetics of donepezil has been demonstrated, the estimated trough concentration was corrected for each patient by the daily donepezil dose.[23]

Genotyping

Genomic DNA was extracted from EDTA blood samples with the FlexiGene DNA extraction kit and the QIAamp DNA Blood Mini Kit (QIAGEN, Hombrechtikon, Switzerland) according to the manufacturer's protocols. The following SNPs were detected by real-time polymerase chain reaction with 5'-nuclease allelic discrimination assays (ABI PRISM 7000; Applied Biosystems, Rotkreuz, Switzerland) according to previous studies or manufacturer's instructions:[17,26] *CYP2D6*3* (rs35742686), *CYP2D6*4* (rs3892097), *CYP2D6*6* (rs5030655), *CYP3A4*1B* (rs2740574), *CYP3A4* rs4646437C>T, *CYP3A5*3* (rs776746), *CYP3A7*1C* (2262T>A and 2270T>G), *POR*28* (rs1057868), *NR1I2* rs1523130, *NR1I2* rs2472677, *NR1I2* rs7643645, *ABCB1* 2677G>T (rs2032582), *ABCB1* 3435C>T (rs1045642), *ABCB1* 1236C>T (rs1128503). Gene deletion (*CYP2D6*5*) and duplication/multiplication (*CYP2D6*xN*) were analyzed by TaqMan copy number assay and long PCR, respectively, as previously described.[26] Internal quality control samples of known genotype were included in all analyses.

Statistical Analysis

Group comparisons were performed with the non-parametric Wilcoxon and Kruskal-Wallis rank sum tests for continuous variables and with the Fisher's exact test for categorical variables. Associations between continuous variables were tested with the Spearman rank-order correlation coefficients (r_s). Multiple regression analyses were performed using the log-transformed value of the dose-adjusted estimated trough concentration as dependent variable whereas age, gender, comedication and genetic polymorphisms were considered as independent variables. The adjusted values of R^2 obtained from the regression models are reported as a measure of the explained variability. Haplotypes were inferred using the R software (version 2.11.1).[27] All tests were two-sided and a P value ≤0.05 was considered statistically significant. Analyses were performed using STATA software (version 11.2; StataCorp, College Station, Texas, USA).

RESULTS

Study population

A total of 129 patients fulfilled the inclusion criteria of one month treatment with a stable daily dose of 5 mg (n=45) or 10 mg (n=84) donepezil. Of these, 127 were taking the drug once daily whereas two were splitting it into two intakes. The sample consisted of 37 (29%) men and 92 (71%) women with a mean (±SD) age of 84±6 years (range 62-99 years). All but one of the participants were Caucasians. The patients were diagnosed with AD (n=85), mixed dementia (n=24), vascular dementia (n=5) and Lewy body dementia (n=2). In 13 subjects, the type of dementia was not specified. The patients received on average (±SD) 7±3 (range 0-13) concomitant drugs. No patient had moderate or severe hepatic impairment. Demographic and clinical characteristics are shown in Table 1.

Donepezil concentration variability and relationship with demographic factors

The calculated trough plasma concentrations ranged from 8-72 ng/ml and 13-105 ng/ml among patients receiving 5 mg and 10 mg donepezil per day, respectively. The estimated trough plasma concentration corrected by the dose ranged between 1.3 and 14.4 ng/ml•mg, with a 11-fold variability (Table 1). Female patients displayed significant higher dose-adjusted plasma concentrations compared to males (median 5.3 versus 4.4 ng/ml•mg, $p<0.001$) (Table 2). Age ($r_s=0.12$, $p=0.2$), smoking ($p=0.4$), elevated alcohol consumption ($p=0.5$) and impaired renal function ($r_s=-0.05$, $p=0.6$) were not significantly associated with dose-adjusted donepezil concentrations.

Six patients (4.7%) were taking strong CYP2D6 inhibitors (paroxetine n=3, levopromazine n=2 and fluoxetine n=1) and two patients (1.6%) strong CYP3A inhibitors (amiodarone n=1, diltiazem n=1).[28,29] All but one of these patients were CYP2D6 EMs. The subject taking fluoxetine as comedication was genotyped as CYP2D6 PM and was, therefore, not considered as receiving a CYP2D6 inhibitor in the statistical analyses. Interestingly, this patient had the highest dose-adjusted donepezil concentration of the group (14.4 ng/ml•mg), a finding that might be related to the additive effect of the CYP2D6 PM

status and the moderate inhibition of CYP3A by fluoxetine. Subjects receiving strong CYP2D6 and CYP3A inhibitors demonstrated higher dose-adjusted donepezil concentrations compared to the rest of the cohort, although the difference was at the limit of significance for CYP2D6 inhibitors (median 6.9 versus 5.0 ng/ml•mg, p=0.059) and not significant for CY3A inhibitors (5.8 versus 5.0 ng/ml•mg, p=0.391) (Table 2). Four patients, among one CYP2D6 PM, taking CYP3A inducers (prednisone n=3, pioglitazone n=1) had lower dose-adjusted plasma concentrations (median 3.8 versus 5 ng/ml•mg, p=0.15), but this difference did not reach statistical significance.

Relationship between genotypes and donepezil plasma concentrations

***CYP2D6* genotypes**

Genotype frequencies are shown in Table 3. Allele and genotype frequencies are in agreement with previous reports in Caucasians.[26,30] CYP2D6 homozygous EMs (homEMs) (n=65) displayed significantly lower dose-adjusted plasma concentrations compared to heterozygous EMs (hetEMs)(n=55) and PMs (n=7) (median 4.7 versus 5.3, p=0.031 and versus 6.3 ng/ml•mg, p=0.026, respectively). Moreover, the UMs (n=2) presented significantly lower dose-adjusted plasma concentrations than homEMs (median 2.6 versus 4.7 ng/ml•mg, p=0.03) (Figure 1, Table 2).

***CYP3A4/5/7*, *POR*, *NR1I2* genotypes**

The observed genotype frequencies are presented in Table 3. They are in agreement with reported results[17,19,31] and no deviation from Hardy-Weinberg equilibrium was observed (p>0.05). In the Caucasian subsample (n=128), linkage disequilibrium was observed between *CYP3A4*1B* and *CYP3A4* rs4646437 (D'=1, r^2=0.18), *CYP3A4*1B* and *CYP3A5*3* (D'=1, r^2=0.32), and between *CYP3A4* rs4646437 and *CYP3A5*3* (D'=0.76, r^2=0.34), which is in line with previous studies.[31] *NR1I2* rs2472677 was moderately linked to *NR1I2* rs1523130 (D'=0.24, r^2=0.05) and *NR1I2* rs7643645 (D'=0.57, r^2=0.27).

No association was found between *CYP3A4/5/7* single nucleotide polymorphisms (SNP) or haplotypes and dose-adjusted donepezil plasma concentrations. Similarly, no influence was observed when the sample was stratified by CYP2D6 metabolizer status to take into account the potential confounding effect of this genotype. In contrast, a tendency toward lower plasma concentrations was observed in patients carrying one or two *POR*28* alleles (p=0.105). When only CYP2D6 EMs were considered, the difference reached statistical significance (median CC 5.2 versus CT/TT 4.7 ng/ml•mg, p=0.038) (Table 2). Interestingly, the effect was more pronounced in CYP2D6 hetEMs than in homEMs (data not shown).

Carriers of the *NR1I2* rs1523130 CC genotype had significantly lower dose-adjusted drug concentrations than carriers of CT/TT genotypes (median 4.5 versus 5.2 ng/ml•mg, p=0.021) (Table 2). The relationship was also observed taking only CYP2D6 EMs into account (p=0.038) (Table 2). Neither *NR1I2* rs2472677 and *NR1I2* rs7643645 polymorphisms nor *NR1I2* haplotypes were associated with donepezil kinetics, even when considering the different *CYP2D6* genotypes.

ABCB1 genotypes

The *ABCB1* genotype frequencies are presented in Table 3. No deviation from Hardy-Weinberg equilibrium was observed (p>0.05). Linkage disequilibrium was noted between *ABCB1* 3435CT and 2677GT (D'=0.80, r^2=0.47), 3435CT and 1236CT (D'=0.75, r^2=0.40) and between 2677GT and 1236CT (D'=0.86, r^2=0.72). Genotype frequencies and linkage disequilibrium are consistent with previous reports.[31,32]

The SNPs *ABCB1* 3435C>T and 2677G>T were not found to be significantly related to dose-adjusted donepezil concentrations (p=0.927 and p=0.141). However, a trend toward lower plasma concentrations was observed in patients carrying the *ABCB1* 1236CT/TT genotypes (median CC 5.6 versus CT/TT 4.8 ng/ml•mg, p=0.084). Moreover, a trend toward higher plasma concentrations was measured in patients carrying the haplotype *ABCB1* 3435T/2677G/1236C (n=29) compared to non-carriers (n=100) (median 5.2 versus 5.0 ng/ml•mg, p=0.084). When the sample was stratified by CYP2D6 EM status, the associations between donepezil concentrations and the *ABCB1* 2677G>T and 1236G>T polymorphisms, as well as the haplotype 3435T/2677G/1236C, reached statistical significance (median 2677G>T GG 5.7 versus GT/TT 4.7 ng/ml•mg, p=0.046; 1236C>T CC 5.7 versus CT/TT 4.7 ng/ml•mg, p=0.024; carriers 3435T/2677G/1236C 5.4 versus non-carriers 4.9 ng/ml•mg, p=0.021) (Table 2).

Multivariate genotype analysis

In multiple regression analyses, the combined effect of different covariates (age, gender, comedication with CYP2D6/CYP3A inhibitors and/or CYP3A inducers) and genotypes on dose-adjusted donepezil concentrations was assessed. Age, female gender and moderate and strong CYP2D6 inhibitors were significantly related to higher dose-adjusted donepezil plasma concentrations (p=0.014, p=0.005, p=0.007, respectively) (Table 4). For CYP3A inducers, a trend toward lower plasma concentrations was observed (p=0.072) while the influence of CYP3A inhibitors was not significant (p=0.287). Moreover, the CYP2D6 genotypes and the SNP *NR1I2* rs1523130 were significantly related to the plasma concentrations (homEMs versus hetEMs, PMs and UMs, p=0.037, p=0.005, p=0.005, respectively, *NR1I2* rs1523130 p=0.005). Neither the investigated polymorphisms in *ABCB1* and *CYP3A4/5/7*, the allele *POR*28* nor the SNPs *NR1I2* rs2472677 and *NR1I2* rs7643645 had a significant impact. The model explained 25% of the variability of dose-adjusted donepezil concentrations.

When excluding CYP2D6 PMs and UMs and considering only CYP2D6 EMs in the model, the covariates age, gender and CYP2D6 inhibitors and the SNP *NR1I2* rs1523130 were significantly related to donepezil plasma concentrations (p=0.048, p<0.001, p=0.009 and p<0.001, respectively), which is in agreement with the multivariate model including all *CYP2D6* genotypes (Table 4). In addition, the relationship of the allele *POR*28* with plasma concentrations was significant in the CYP2D6 EM subgroup (p=0.018), which is in line with the univariate analysis. The model explained 22% of the variability of dose-adjusted donepezil concentrations in CYP2D6 EMs.

Furthermore, multiple regression analyses were performed including the haplotype *ABCB1* 3435T/2677G/1236G instead of the *ABCB1* polymorphisms. The influence of the haplotype was significant in the CYP2D6 EM subgroup analysis (p=0.024), but not in the whole sample (p=0.081), which is in agreement with the univariate analyses. Both models explained 26% of the variability of dose-adjusted donepezil concentrations.

Finally, the proportions of explained variability by models including all analyzed genetic polymorphisms and models including only CYP2D6 genotypes were compared. The explained variabilities were only slightly higher in the complete models with values of 25% (all genetic polymorphisms and covariates) versus 23% (CYP2D6 genotypes and covariates) and, excluding the covariates, with 8% (all genetic polymorphisms) versus 6% (CYP2D6 genotypes). This suggests that the functional alleles of *CYP2D6* accounted for the major part of the genetically determined variability in donepezil pharmacokinetics.

Relationship between plasma concentration and genotypes on adverse events (AE)

57% of the patients reported no AE while 43% experienced at least one AE. Among them 14% had gastrointestinal problems (nausea, vomiting, diarrhea, anorexia), 16% dizziness, 11% headache, 8% insomnia, 20% fatigue, 6% muscle cramp, 13% accidents (falls) and 11% pain. No difference in frequencies was seen between patients receiving 5 mg and 10 mg donepezil per day. No significant association was found between donepezil plasma concentrations and the presence of AEs. However, when CYP2D6 PMs or patients with strong CYP2D6 inhibitors were compared with the rest of the cohort, the former had significantly more AEs (75% (n=9) versus 40% (n=46) ≥1 AE, p=0.029), which might be due to the trend toward higher donepezil trough plasma concentrations in this group (median (IQR) 55 (40-68) versus 41 (25-56) ng/ml, p=0.078). Additionally, a tendency toward more AEs was observed in carriers of the *ABCB1* 3435T/2677G/1236C haplotype (59% (n=17) versus 38% (n=38) with ≥1 AE, p=0.059), which might as well be due to higher donepezil trough plasma concentrations (median (IQR) 50 (30-63) ng/ml versus 41 (25-56) ng/ml, p=0.102).

DISCUSSION

There is a large interindividual variability in the response to acetlycholinesterase inhibitors, with responder rates as low as 15-35%.[3,33,34] Since a concentration-response effect has been reported for donepezil,[35] differences in therapeutic outcome could be related, to some extent, to the drugs' plasma concentrations. In this study, an 11-fold variability in dose-adjusted donepezil plasma concentrations was observed, which confirmed the results of a previous report.[4]

The *CYP2D6* genotype appeared to be the major genetic factor influencing dose-adjusted donepezil plasma concentrations. In the study sample, 7 CYP2D6 PMs, 55 hetEMs, 65 homEMs and 2 UMs were included. Even though considerable overlaps were measured between the groups, hetEMs and PMs demonstrated significantly higher dose-adjusted plasma concentrations than homEMs, with a

13% and 34% increase in the median values. Moreover, the two UMs had lower dose-adjusted plasma concentrations than the median value of homEMs. Although *CYP2D6* genotypes have been shown to influence the plasma concentrations of many drugs,[8,36] only one previous study, to our knowledge, examined the relationship between *CYP2D6* genotypes and donepezil plasma concentrations.[4] Results showed a tendency toward lower plasma concentrations in homEMs compared to hetEMs, and in UMs compared to EMs,[4] but the limited sample size (n=42) and statistical power precluded a formal conclusion. The current study broadens our knowledge in showing for the first time these significant relationships.

Further accentuating the importance of CYP2D6 in donepezil metabolism, a 1.4-fold increase in plasma concentrations was measured in patients receiving strong inhibitors of CYP2D6. In the multivariate analysis, even moderate CYP2D6 inhibitors were significantly related to higher dose-adjusted donepezil concentrations. The clinical significance of these interactions remains to be determined, but careful monitoring of these patients might be beneficial.

In contrast to CYP2D6, genetic variations in *CYP3A* do not appear to play a significant role as our results showed no associations between *CYP3A4/5/7* genetic polymorphisms and dose-adjusted donepezil concentrations. These results are consistent with a previous study analyzing polymorphisms in *CYP3A4/5* and the fact that the current knowledge of genetic variations in *CYP3A4/5/7* only marginally explains the large interindividual variability in CYP3A activity.[37]

To our knowledge, the present study is the first to examine the relationship between POR, PXR, and donepezil pharmacokinetics. Interest in the electron transferring enzyme POR and the nuclear receptor PXR resulted from the search on polymorphisms of genes influencing CYP3A expression and activity. The most common studied *POR* polymorphism is the exonic SNP rs1057868C>T, defining the allele **28*. Our group showed the influence of this polymorphism on CYP3A activity in two independent cohorts, with *POR*28* TT carriers presenting a 1.6-fold higher CYP3A activity than carriers of the C allele.[17] In line with these findings, the current study demonstrates that patients carrying at least one *POR*28* allele have a tendency toward lower dose-adjusted donepezil plasma concentrations. In CYP2D6 EMs, the difference reached statistical significance, which suggests a confounding of the effect by CYP2D6 PMs and UMs when considering the whole cohort. Moreover, stratification of the sample by *CYP2D6* genotype revealed a pronounced effect of the *POR*28* in hetEMs. The decreased CYP2D6 activity in hetEMs, partly compensated by CYP3A, might explain the distinct impact of the *POR*28* in these subjects.

Recently, attention was drawn on three polymorphisms in the regulatory region of *NR1I2*, which were associated with PXR and CYP3A4 expression *in vitro*, namely rs1523130, rs2472677 and rs7643645. In this study, a significant association of the *NR1I2* rs1523130 was found with dose-adjusted donepezil concentrations, with patients carrying at least one T allele having higher plasma concentrations compared to subjects carrying two C alleles. *In vitro* studies demonstrated decreased CYP3A4 mRNA concentrations in liver tissue with the *NR1I2* rs1523130 CT/TT genotypes.[19,20] Therefore, the observation of higher donepezil concentrations in *NR1I2* rs1523130 CT/TT carriers

could be due to a lower CYP3A4 activity. Because PXR mediates the induction of several phase I and II drug-metabolizing enzymes as well as transporters including P-pg, the influence of the *NR1I2* rs1523130 might also be mediated by other factors than CYP3A expression.

Finally, the three common P-gp polymorphisms were investigated: *ABCB1* 3435C>T, 2677G>T and 1236C>T. Although these SNPs were tested in many *in vitro* and *in vivo* studies, the literature bears no consensus on the phenotypic associations with these SNPs.[16] A trend toward lower dose-adjusted plasma concentrations was found in T allele carriers of the *ABCB1* 2677G>T and 1236C>T SNPs, but was no more significant in the multivariate analysis. Interestingly, carriers of the haplotype *ABCB1* 3435T/2677G/1236C showed higher plasma concentrations compared to non-carriers, however, the difference reached statistical significance in the multivariate analysis only if CYP2D6 EMs were taken into account. Results of a recent study showed a tendency toward lower donepezil plasma concentrations in patients carrying two alleles of the haplotype 3435T/2677T/1236T. However, the difference did not reach statistical significance,[14] and could not be confirmed by the present results. It should be mentioned that P-gp polymorphisms might influence donepezil plasma concentrations through altered absorption, distribution and/or excretion. On the other hand, it has also been shown that individuals homozygous for the *ABCB1* 2677 T allele have enhanced constitutive CYP3A4 expression in liver and intestine compared with those homozygous for the G allele.[38] This difference in CYP3A activity might therefore explain the higher donepezil dose-adjusted plasma concentrations in carriers of the *ABCB1* 2677 G allele and of the haplotype *ABCB1* 3435T/2677G/1236C.

Overall, 43% of the patients reported at least one AE when receiving 5 mg or 10 mg donepezil daily. This prevalence is lower than in other studies, where incidences of 65% (5 mg/day) and 83% (10 mg/day) have been reported.[2] This discrepancy might be due to the fact that most of the patients included in the present study had already been treated for several months with donepezil. Therefore, at inclusion the transient AEs were no more present and intolerant patients would already have discontinued the treatment and be ineligible. Because of the higher incidence of AEs in patients treated with 10 mg donepezil daily, a relationship between plasma concentrations and frequency of AEs has been suggested.[2] In our study population, the frequency of subjects experiencing at least one AE was significantly higher in CYP2D6 PMs or patients taking comedications which are strong CYP2D6 inhibitors. This could be due to the higher trough concentrations measured in these subjects, even though the difference did not reach statistical significance. Furthermore, a similar trend toward a higher incidence of AEs was noticed in patients carrying the haplotype *ABCB1* 3435T/2677G/1236G, also displaying slightly higher trough concentrations. These results suggest that future prospective studies should be performed to determine whether therapeutic monitoring of donepezil could be useful to reduce AEs.

This naturalistic cross-sectional study has several limitations. The patients included in the study are very heterogeneous thus confounding by different factors, such as comobidities or comedication, cannot be excluded. Blood sampling for plasma concentration determination was performed at any time of the day, thus, trough plasma concentrations had to be estimated in order to be able to compare the patients. Because of the long half-life of the drug (70 hours), resulting in small differences

between peak and trough concentrations, our estimation of the trough concentrations is considered to be suitable for the objectives of the study. Prevalent AEs were recorded at inclusion and, as previously mentioned, most patients were already receiving donepezil for several months. This might have complicated differentiating between donepezil related AEs and effects of concomitant diseases or medication. Considering these limitations, confirmation of the presented findings in other study cohorts is required.

In summary, the present study shows a large interindividual variability in donepezil plasma concentrations. Functional alleles of *CYP2D6* accounted for a major part of the genetically determined variability. However, genetic variations in *POR*, *NR1I2* and *ABCB1* were also related to donepezil pharmacokinetics. These findings are an important contribution to a better understanding of donepezil pharmacokinetics and, potentially, clinical effectiveness and tolerance.

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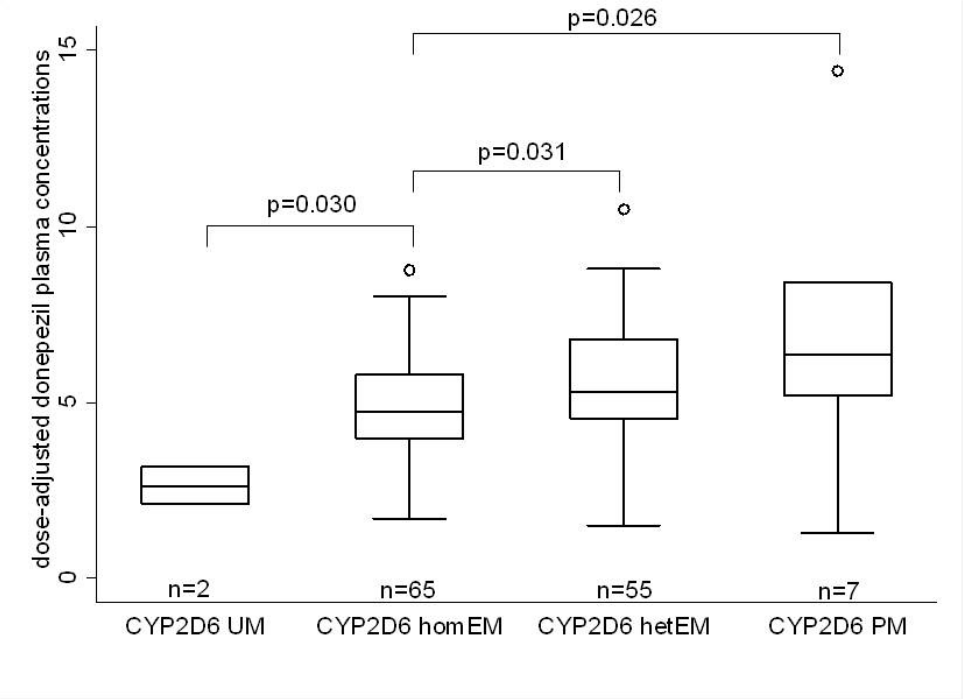

Figure 1: Influence of the CYP2D6 genotype on dose-adjusted donepezil plasma concentrations. In the box plots median and interquartile ranges are shown.

Table 1: Characteristics of the study population (n=129)

Characteristic	Category			
Age (years)		(mean±SD; range)	84 ± 6	62-99
Gender	male	(n; %)	37	28.6
	female	(n; %)	92	71.3
Duration of treatment (years)[a]		(mean±SD; range)	2 ± 1.7	0.1-8
Smoking		(n; %)	9	7.0
Alcohol[b]		(n; %)	3	2.3
Renal impairment	eGFR[c] ≥ 60	(n; %)	59	45.7
	eGFR[c] 30-59	(n; %)	67	51.9
	eGFR[c] <30	(n; %)	3	2.3
Dose per day	5 mg	(n; %)	45	34.9
	10 mg	(n; %)	84	65.1
Estimated C_0[d] (ng/ml)	5 mg	(median (IQR); range)	23 (19-29)	8-72
	10 mg	(median (IQR); range)	52 (43-64)	13-105
C_0/D[e] (ng/ml•mg)		(median (IQR); range)	5.0 (4.1-6.2)	1.3-14.4
Comedication				
CYP2D6 inhibitors	moderate	(n; %)	65	50.4
	strong	(n; %)	6[f]	4.7
CYP3A inhibitors	moderate	(n; %)	37	28.7
	strong	(n; %)	2	1.6
CYP3A inducers		(n; %)	4	3.1

a) n=120, in 9 subjects the duration of treatment is unknown
b) >40 g per day in men and >20 g per day in women
c) Estimated glomerular filtration reat with MDRD (Modification of Diet in Renal Disease) formula in ml/min/1.73m^2 [39]
d) C_0: trough plasma concentration
e) C_o corrected by the daily dose
f) Includes one CYP2D6 PM who was not considered as receiving a CYP2D6 inhibitor in the statistical analyses.

Table 2: Univariate analyses of the influence of demographic factors and genotypes on dose-adjusted donepezil concentrations

			all CYP2D6 genotypes			CYP2D6 EMs		
			n	Dose-adjusted plasma level[a]	P values[b]	n	Dose-adjusted plasma level[a]	P values[b]
Demographic factors		**category**						
Gender		male	37	4.4 (3.7-4.9)	>0.001			
		female	92	5.3 (4.3-6.5)				
Strong CYP2D6 inhibitors		yes	5	6.9 (5.8-7.6)	0.059			
		no	124	5.0 (4.0-6.1)				
Strong CYP3A inhibitors		yes	2	5.8 (5.8-5.8)	0.391			
		no	127	5.0 (4.0-6.3)				
CYP3A inducers		yes	4	3.8 (2.4-5.2)	0.150			
		no	125	5.0 (4.1-6.2)				
Genetic factors		**genotype**						
CYP2D6								
		UM	2	2.6 (2.1-3.2)	0.030[c]			
		homEM	65	4.7 (4.0-5.8)				
		hetEM	55	5.3 (4.5-6.8)	0.031[c]			
		PM	7	6.3 (5.2-8.4)	0.026[c]			
CYP3A4								
	allele *1B	*1/*1	123	5.0 (4.1-6.2)	0.878	116	5.0 (4.1-6.1)	0.804
		*1/*1B	6	4.6 (3.2-8.8)		4	4.6 (3.6-7.0)	
	rs4646437	CC	103	5.0 (4.1-6.3)	0.603	99	5.0 (4.1-6.2)	0.344
		CT	26	4.7 (4.0-6.2)		21	3.6 (4.4-5.3)	
CYP3A5								
	allele *3	*1/*3	16	5.0 (4.2-6.3)	0.803	15	5.0 (4.5-6.2)	0.756
		*3/*3	113	5.0 (4.1-6.2)		106	5.0 (4.1-6.1)	
CYP3A7								
	allele **1C*	*1/*1	123	5.0 (4.1-6.3)	0.712	114	5.0 (4.1-6.1)	0.756
		*1/*1C	5	5.0 (4.4-6.2)		5	5.0 (4.4-6.2)	
		*1C/*1C	1	3.8		1	3.8	
POR								
	allele **28*	CC	74	5.2 (4.2-6.6)	0.105	69	5.2 (4.6-6.6)	0.038
		CT	46	4.8 (3.9-6.0)		42	4.7 (3.9-5.8)	
		TT	9	4.6 (3.5-5.8)		9	4.6 (3.5-5.8)	
NR1I2								
	rs1523130	CC	47	4.5 (3.7-5.8)	0.021	43	4.5 (3.8-5.8)	0.038
		CT	56	5.2 (4.4-6.7)		52	5.0 (4.4-6.1)	
		TT	26	5.2 (4.2-6.4)		25	5.3 (4.5-6.4)	
	rs2472677	TT	50	5.0 (4.0-5.9)	0.691	47	5.0 (4.2-5.9)	0.996
		TC	67	5.0 (4.1-6.4)		62	5.0 (4.1-6.2)	
		CC	12	5.0 (4.1-7.2)		11	5.0 (4.1-6.9)	
	rs7643645	AA	61	5.1 (4.2-6.3)	0.718	55	5.0 (4.2-6.3)	0.658
		AG	56	5.0 (4.1-6.1)		54	5.0 (4.1-6.0)	
		GG	12	4.6 (3.7-7.2)		11	4.2 (3.7-6.9)	

ABCB1								
	3436C>T	CC	36	5.2 (3.8-6.3)	0.927	35	5.3 (4.0-6.3)	0.603
		CT	55	4.9 (3.9-6.1)		54	4.9 (3.9-6.1)	
		TT	38	5.2 (4.3-6.4)		31	4.8 (4.3-5.9)	
	2677G>T	GG	45	5.6 (4.1-6.4)	0.141	44	5.7 (4.3-6.6)	0.046
		GT	56	4.8 (3.9-5.7)		54	4.8 (4.0-5.7)	
		TT	28	5.0 (4.3-6.5)		26	4.7 (4.3-5.8)	
	1236C>T	CC	45	5.6 (4.4-6.8)	0.084	44	5.7 (4.5-6.9)	0.024
		CT	58	4.7 (3.9-5.5)		56	4.7 (3.9-5.5)	
		TT	26	5.3 (4.2-6.2)		20	5.0 (4.1-5.8)	
	3435T/2677G/ 1236C	carriers	29	5.2 (4.5-7.1)	0.084	92	4.9 (3.9-5.8)	0.021
		non-carriers	100	5.0 (3.9-6.0)		28	5.4 (4.6-7.1)	

a) Dose-adjusted plasma levels are expressed as median (interquartile range) in ng/ml•mg

b) P values determined by Wilcoxon test; dominant model grouped by the presence of at least one variant allele versus wild type

c) P value refers to homozygous extensive metabolizers (homEM) versus ultrarapid metabolizers (UM), heterozygous extensive metabolizers (hetEM) and poor metabolizers (PM), respectively.

Table 3: Genotype frequencies in the whole cohort (n=129)

	n	Frequency (%)	95% CI (%)		n	Frequency (%)	95% CI (%)		n	Frequency (%)	95% CI (%)
***ABCB1* 3435CT**				***CYP3A4 *1B***				***NR1I2* rs2472677**			
CC	36	28	20-36	*1/*1	123	95	90-98	TT	50	39	30-48
CT	55	43	34-52	*1/*1B	6	5	2-10	TC	67	52	43-61
TT	38	29	22-38					CC	12	9	5-16
***ABCB1* 2677GT**				***CYP3A5 *3***				***NR1I2* rs7643645**			
GG	45	35	27-44	*1/*3	16	12	7-19	AA	61	47	38-56
GT	56	43	35-52	*3/*3	113	88	81-93	AG	56	44	35-52
TT	28	22	15-30					GG	12	9	5-16
***ABCB1* 1236CT**				***CYP3A7 *1C***				***POR*28***			
CC	45	35	27-43	*1/*1	123	95	90-98	CC	74	57	48-66
CT	58	45	36-54	*1/*1C	5	4	1-9	CT	46	36	27-45
TT	26	20	14-28	*1C/*1C	1	1	-	TT	9	7	3-13
***CYP3A4* rs4646437**				***NR1I2* rs1523130**				***CYP2D6***[a]			
CC	103	80	72-86	CC	47	37	*28-45*	PM	7	5	2-11
CT	26	20	14-28	CT	56	43	*35-52*	hetEM	55	43	34-52
				TT	26	20	*14-28*	homEM	65	50	41-59
								UM	2	2	0-6

a) CYP2D6: PM**,** poor metabolizer: *4/*4 n=5, *4/*5 n=2; hetEM**,** heterozygous extensive metabolizer: *1/*3 n=6, *1/*4 n=36, *1/*5 n=9 ; *1/*6 n=4; homEM, homozygous extensive metabolizer: *1/*1 n=61, *4/*xN n=3, *6/*xN n=1; UM, ultrarapid metabolizer: *1/*XN or *XN/*XN, n=2.

Table 4: Multivariate analyses of the influence of genotypes on dose-adjusted donepezil concentrations in all *CYP2D6* genotypes and in CYP2D6 EMs

		all CYP2D6 genotypes		CYP2D6 EM	
		Coefficient (SE)[a]	***P* value[a]**	**Coefficient (SE)[a]**	***P* value[a]**
Demographic factors					
Age		0.012 (0.005)	0.014	0.010 (0.005)	0.048
Female Gender		0.197 (0.068)	0.005	0.258 (0.063)	<0.001
CYP2D6 inhibitors		0.178 (0.065)	0.007	0.157 (0.059)	0.009
CYP3A inhibitors		0.071 (0.067)	0.287	0.092 (0.062)	0.138
CYP3A inducers		-0.330 (0.182)	0.072	0.231 (0.192)	0.231
Genetic factors					
CYP2D6	homEM versus hetEM	0.130 (0.061)	0.037		
	homEM versus PM	0.417 (0.145)	0.005		
	hom EM versus UM	-0.721 (0.252)	0.005		
CYP3A4	allele **1B*	0.051 (0.178)	0.774	-0.026 (0.180)	0.887
	rs4646437	0.018 (0.091)	0.846	-0.032 (0.090)	0.718
CYP3A5	allele **3*	-0.050 (0.124)	0.687	-0.098 (0.116)	0.400
CYP3A7	allele **1C*	0.014 (0.142)	0.919	-0.073 (0.130)	0.575
POR	allele **28*	-0.050 (0.060)	0.401	-0.136 (0.056)	0.018
NR1I2	rs1523130	0.185 (0.064)	0.005	0.234 (0.062)	<0.001
	rs2472677	-0.039 (0.107)	0.720	-0.033 (0.101)	0.749
	rs7643645	-0.034 (0.062)	0.577	-0.002 (0.058)	0.979
ABCB1	3436C>T	0.089 (0.101)	0.377	0.071 (0.093)	0.447
	2677G>T	-0.207 (0.160)	0.198	-0.156 (0.145)	0.285
	1236C>T	0.059 (0.141)	0.676	0.064 (0.129)	0.619
	Intercept	0.265 (0.462)	0.568	0.531 (0.450)	0.241

a) Coefficients and P values obtained from the linear multiple regression analysis using the log-transformed dose-corrected donepezil trough concentrations as dependent variable and genotypes, age, gender, comedication with CYP2D6 inhibitors, CYP3A inhibitors and CYP3A inducers as independent variables.

4.2 Article V: Population pharmacokinetic study of memantine: effects of clinical and genetic factors

Summary

In this article, the results of the pharmacogenetic study in patients treated with memantine are described. Memantine is mainly eliminated unchanged by the kidneys with implication of active renal secretion by cation transporters. Moreover, considerable inter-individual variability in plasma concentrations has been reported in previous studies. A population pharmacokinetic study, using NONMEM, was performed, which included clinical and genetic data from 108 patients. Common polymorphisms in renal cation transporters (*SCL22A1/2/5*, *SLC47A1*, *ABCB1*) and nuclear receptors (*NR1I2*, *NR1I3*, *PPAR*) involved in transporter expression were investigated. An average clearance of 5.2 l/h with a 27% inter-individual variability (CV%) was found. In line with the primarily renal elimination, the glomerular filtration rate markedly influenced memantine clearance. Moreover, female patients showed a slower elimination with a 20% lower clearance. No significant relationship between genetic variations in cation transporter genes and memantine clearance was found. In contrast, the polymorphism *NR1I2* rs1523130 was significantly associated with memantine clearance, with carriers of the *NR1I2* rs1523130 CT/TT genotypes presenting a 16% slower memantine elimination than carriers of the CC genotype. To date, this is the first study that comprehensively evaluated the effect of polymorphisms in renal transporters and nuclear receptors on memantine pharmacokinetics. The better understanding of inter-individual variability in memantine pharmacokinetics might be beneficial in the context of individual dose optimization.

Article submitted

POPULATION PHARMACOKINETIC STUDY OF MEMANTINE: EFFECTS OF CLINICAL AND GENETIC FACTORS

Muriel Noetzli[1], Monia Guidi[2], Karsten Ebbing[3], Stephan Eyer[4], Laurence Wilhelm[5], Agnès Michon[6], Valérie Thomazic[6], Abdel-Messieh Alnawaqil[7], Sophie Maurer[1], Serge Zumbach[7], Panteleimon Giannakopoulos[8], Armin von Gunten[3], Chantal Csajka[2,9], Chin B. Eap[1,9]

1. Unit of Pharmacogenetics and Clinical Psychopharmacology, Centre for Psychiatric Neuroscience, Department of Psychiatry, University Hospital Lausanne, Prilly-Lausanne, Switzerland.
2. Division of Clinical Pharmacology and Toxicology, Department of Medicine, University Hospital Lausanne, Lausanne, Switzerland
3. Service of Old Age Psychiatry, Department of Psychiatry, University Hospital Lausanne, Prilly-Lausanne, Switzerland.
4. Geriatric Medicine and Geriatric Rehabilitation, Department of medicine, University Hospital Lausanne, Lausanne, Switzerland.
5. Fondation Nant, Consultation Aigle, Aigle, Switzerland.
6. Division of Geriatric Psychiatry, Department of Psychiatry, University Hospital Geneva, Geneva, Switzerland
7. Fribourg Mental Health Network, Department of Psychiatry, Cantonal Hospital Fribourg, Marsens, Switzerland.
8. Divisions of General Psychiatry, Department of Mental Health and Psychiatry, University Hospital Geneva, Geneva, Switzerland.
9. Department of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Boulevard d'Yvoy 20, 1211 Geneva 4, Switzerland.

Author contributions

Study concept and design: C.B. Eap

Genetic analyses: M. Noetzli, S. Maurer

Population pharmacokinetic analyses: M. Guidi, C. Csajka

Data interpretation: M. Noetzli, M. Guidi, C.B. Eap, C. Csajka

Drafting of the manuscript: M. Noetzli, M. Guidi

Patient recruitment and study supervision: M. Noetzli, K. Ebbing, S. Eyer, L. Wilhelm, A. Michon, V. Thomazic, A.M. Alnawaqil, S. Maurer, S. Zumbach, P. Giannakopoulos, A. von Gunten, C.B. Eap

Critical revision of the manuscript for important intellectual content: all authors

ABSTRACT

Memantine, a frequently prescribed antidementia drug, is mainly eliminated unchanged by the kidneys, partly *via* tubular secretion. Considerable interindividual variability in plasma levels has been reported. To investigate clinical and genetic factors influencing memantine disposition, a population pharmacokinetic study was performed including data from 108 patients recruited in a naturalistic setting. Patients were genotyped for common polymorphisms in renal cation transporters (*SCL22A1/2/5*, *SLC47A1*, *ABCB1*) and nuclear receptors (*NR1I2*, *NR1I3*, *PPAR*) involved in transporter expression. The average clearance was 5.2 l/h with a 27% interindividual variability (CV%). Glomerular filtration rate (p=0.007) and gender (p=0.001) markedly influenced memantine clearance. *NR1I2* rs1523130 was identified as the unique significant genetic covariate for memantine clearance (p=0.006), with carriers of the *NR1I2* rs1523130 CT/TT genotypes presenting a 16% slower memantine elimination than carriers of the CC genotype. The better understanding of interindividual variability might be beneficial in the context of individual dose optimization.

INTRODUCTION

Memantine is a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist, which is indicated for the treatment of moderate to severe Alzheimer's disease. Memantine has generally been shown to be a well-tolerated drug,[1] the most common adverse events (AE) include dizziness, somnolence, headache and constipation.[1,2] The major part of an administered memantine dose is excreted unchanged by the kidneys (75-90%) with the remaining memantine converted to numerous metabolites.[3] The total renal clearance (CL) substantially exceeds glomerular filtration rate, indicating that a significant part of memantine is eliminated via active tubular secretion by renal transporters.[3] Steady-state plasma levels of 70-150 ng/ml with large interindividual variations have been reported in patients receiving the recommended daily dose of 20 mg.[2] Moreover, a relationship between clinical outcome and frequency of AEs with memantine plasma levels has been suggested.[4]

There is growing evidence that, for some drugs, genetic variations in membrane transporters contribute to interpatient variability in disposition and/or response.[5] Memantine is a known substrate of the human organic cation transporter 2 (OCT2).[6] However, as an organic cation at physiological pH, memantine is probably also handled by other transporters of the renal organic cation transport system, including the organic cation transporters (OCT1-3), the carnitine transporters (OCTN1-3), the multidrug and toxin extrusion proteins (MATE1-2) and the P-glycoprotein (P-gp).[7] Numerous polymorphisms have been described in their respective genes *SLC22A1-3*, *SLC22A4-5*, *SLC47A1-2* and *ABCB1*, some of them are known to alter protein function and expression.[8-13] To our knowledge, no studies investigating the influence of genetic variants in cation transporters on memantine pharmacokinetics have been published. However, several single nucleotide polymorphisms (SNP) in *SLC22A1/2* and *SLC42A1* have been suggested to affect metformin CL.[13,14] Similar effects might be anticipated for memantine, as metformin is an organic cation drug that is mainly eliminated unchanged by the kidneys with implication of active secretion by renal transporters. In addition, *in vitro* and *in vivo*

studies have demonstrated the involvement of the following nuclear receptors in gene expression regulation of cation transporters in different tissues: pregnane X receptor (PXR), constitutive androstane receptor (CAR) and peroxisome proliferator-activated receptor (PPAR).[15-19] There is emerging evidence that polymorphisms in nuclear receptors influence the expression of cytochrome P450 (CYP) drug metabolizing enzymes,[20,21] thus affecting pharmacokinetics of drugs. A similar effect of these polymorphisms on renal drug transporter expression might therefore be hypothesized, which could potentially lead to interindividual variability in drug elimination. Thus, the identification of clinical and genetic factors underlying the interindividual variability in memantine pharmacokinetics may help to improve the understanding of variation in drug response.

The aim of the present study was to quantify the effects of genetic variations in genes of renal cation transporters and nuclear receptors, and of other clinical factors, on memantine plasma levels. In addition, simulations were performed to predict and compare the average steady-state plasma levels (C_{av}) associated with 20 mg, 15 mg and 10 mg once daily dosage regimens for relevant patients' characteristics. Finally, an analysis of the memantine concentration-toxicity relationship was conducted.

RESULTS

A total of 108 patients were included in the study, receiving a daily memantine dose of 5 mg (n=5), 10 mg (n=24), 15 mg (n=4) or 20 mg (n=75). The average (±SD) duration of memantine treatment was 1.8 (±1.7) years (range 1 month to 10 years). Memantine plasma levels measured at steady-state ranged from 11 to 314 ng/ml. The characteristics of the study population are presented in Table 1.

Structural model

A one-compartment model with first-order absorption from the gastro-intestinal tract fitted the data at best. Owing to few data at early time points after drug intake, rendering the estimation of the absorption rate constant k_a imprecise, k_a was fixed to the mean of the bootstrap resampling analysis (0.3 h^{-1} CI_{95}:0.04-1.11^{-1}). In addition to CL, interpatient variability on the volume of distribution of the central compartment (V) and k_a did not further improve the description of the data (change in the objective function (ΔOF) ≤0.9; p>0.3). The estimates and the variability (CV%) of the basic pharmacokinetic model were: CL 5.2 l/h (27%), *V* 172.8 L and k_a 0.3 h^{-1}.

Influence of non-genetic covariates

Table 2 summarizes the model-building steps performed for the covariate analysis. In univariate analyses, sex, age and creatinine CL (CL_{creat}) were identified as significant covariates on memantine CL (ΔOF) ≤-7; p<0.01). Ethnicity, memantine formulation, smoking, grapefruit juice (P-gp inhibitor) or elevated alcohol consumption were not associated with memantine CL (ΔOF≥-12.7; p>0.1). Interacting medications were classified as drugs eliminated via tubular secretion, OCT inhibitors, PXR agonists, and P-gp inhibitors or inducers (Supplementary Table 1). Among those, P-gp inhibitors (ΔOF=-11;

p<0.01) and PXR agonists (ΔOF=-5; p<0.05) increased memantine CL by about 20%. Since the classification of drugs as P-gp inhibitors and PXR agonists was mainly based on *in vitro* data, the clinical significance for pharmacokinetic drug interactions at therapeutic concentrations is unknown. Therefore, the analyses on these covariates were considered to be exploratory and they were not kept in the reported final model to avoid bias. Nevertheless, similar results were found with and without these covariates (data not shown). The model incorporating all relevant non-genetic covariates was built up and further refined by setting them one by one to their null values. This step eliminated the influence of age on CL, but sex and CL_{creat} remained statistically significant (ΔOF=-17.6; p<0.001) and explained altogether 7% of the interindividual variability on CL.

Influence of genetic covariates

A total of 27 polymorphisms in renal transporters and nuclear receptors were analyzed (Table 3). The minor allelic frequencies are in line with the reported frequencies for Caucasians in the SNP database of the National Center for Biotechnology Information.[22] No deviation from Hardy-Weinberg equilibrium was observed (p>0.08), with the exception of CAR rs4073054 (p=0.04).

No significant relationship was found between the investigated SNPs in the genes *SLC22A1/2/5*, *SLC47A1* and *ABCB1* of renal transporters and memantine CL. In contrast, univariate analyses testing the effects of polymorphisms in the nuclear receptors *NR1I2*, *NR1I3*, *RXRA*, *PPARGC* and *PPARG* revealed a significant association of the SNP *NR1I2* rs1523130 with memantine CL (rich model Eq 2 ΔOF=-6.3; p<0.05), explaining 9% of its interindividual variability.

Several models were used to test the relationships between the *NR1I2* rs1523130 genotypic groups and memantine CL (Table 2). Compared to the rich model, linear, power, square root or square models provided similar description of the data using an activity score (Eq 3, 4, 5 and 6 ΔOF<-4; p<0.05). Furthermore, no statistical significant difference in CL in *NR1I2* rs1523130 CT and TT genotypic groups was observed compared to the other models (ΔOF=0.5; p=0.5). CL was 5.87 l/h in *NR1I2* rs1523130 CC individuals and reduced to 4.96 l/h in carriers of the CT or TT alleles.

The final model included sex, CL_{creat} and *NR1I2* rs1523130 polymorphism on memantine CL (ΔOF=-25; p<0.001). These covariates explained 17% of the overall variability in memantine CL. The final model parameters' estimates, together with their bootstrap estimations, are given in Table 4. Figure 1 shows the dose-normalized concentration-time plots of memantine with their 95% prediction intervals.

Simulations

Model-based simulations were performed to evaluate the impact of genetic polymorphism and impaired renal function on memantine C_{av} and to compare these concentrations to the suggested therapeutic range of 90-150 ng/ml.[23]

Individuals with normal renal function carrying the *NR1I2 rs1523130* CT/TT genotypes had a modest 19% increase in median C_{av} after daily doses of 20 mg, with a mean C_{av} increasing from 115 ng/ml (CI95% 55-174 ng/ml) to 137 ng/ml (CI95% 66-208 ng/ml). The model predicted an additional 28% increase in C_{av} in individuals with impaired renal function (CL_{creat} 30 ml/min) in combination with the genetic polymorphism, leading to a mean C_{av} of 169 ng/ml (CI95% 82-256 ng/ml). While considering

the interindividual variability in CL, it is expected that 13%, 32%, 36% and 63% of individuals would exceed the 150 ng/ml upper limit in *NR1I2* rs1523130 CC individuals with normal renal function, CT/TT carriers with normal renal function, CC individuals with reduced renal function and CT/TT carriers with reduced renal function, respectively.

Additionally, simulations were performed to evaluate which dose adjustment would be required in patients with a CL_{creat} of 30 ml/min under assumption of reference alleles for *NR1I2*. For daily doses of 10 mg and 15 mg, the respective predicted mean C_{av} were 38% and 8% inferior to patients with normal renal function receiving 20 mg memantine per day (71 ng/ml and 106 ng/ml vs. 115 ng/ml). Moreover, while accounting for interindividual variability, the dose reductions would lead to C_{av} below the 90 ng/ml limit in 84% and 30% of the individuals after 10 mg and 15 mg daily, respectively. Figure 2 shows the average and 95% predicted interval (PI) of C_{av} obtained for memantine 10 mg, 15 mg and 20 mg daily regimens under various assumptions of *NR1I2* rs1523130 genotypes and renal function.

Concentration-side effect relationships

A total of 54% (n=58) patients reported no AE while 46% (n=50) experienced at least one AE. Constipation was present in 25%, dizziness in 14%, headache in 13% and somnolence in 17% of the patients. Logistic regression analyses between C_{av} or area under the curve ($AUC_{0-\tau}$) and the presence/absence of AEs revealed no significant relationship. In contrast, chi-square analyses between dichotomized C_{av} values and the presence/absence of AEs showed that patients with C_{av} above 150 ng/ml had a significantly higher risk to experience at least one AE (38% (n=26) C_{av}<150 ng/ml vs. 60% (n=24) C_{av}>150 ng/ml, p=0.028). Regarding specific AEs, constipation (16% vs. 40%, p = 0.006) and dizziness (7% vs. 25%, p=0.01) were significantly related to the 150 ng/ml cut-off value, whereas other AEs (headache p = 0.095) and somnolence (p=0.075) were non-significant.

DISCUSSION

Comorbidities, polypharmacy and non-compliance are particular problems in elderly patients with cognitive impairment. It would therefore be of special importance for this population to avoid multiple medication changes due to non-response or side effects. Thus, optimization of memantine treatment through an individual dose-adaption might be beneficial. In this naturalistic cross-sectional study, 108 dementia patients were included receiving a treatment with memantine. A population pharmacokinetic model was developed, integrating genetic and non-genetic factors, to study the interindividual variability in memantine pharmacokinetics. To our knowledge, this is the first pharmacogenetic study on memantine. The CL estimate and its variability are in good accordance with a previous population pharmacokinetic study.[24] As expected, renal function had a marked impact on memantine elimination. The dominant influence of the renal function is in agreement with the extensive renal elimination of memantine and has previously been shown in a single dose study in elderly volunteers.[25] Age and sex were also correlated to memantine elimination, but their influence was integrated in the MDRD (Modification of Diet in Renal Disease) formula used to estimate CL_{creat}.[26] However, the influence of

sex remained beyond correction, which could be related to a lower OCT expression in female, as observed in rats and mice.[27,28]

It is suggested that comedication eliminated via renal tubular secretion or inhibiting organic cation transporters could lead to pharmacokinetic interactions with memantine.[3] To date, only few clinical trials addressed this issue. On one hand, one study reported a decreased memantine CL in patients taking comedication eliminated *via* renal tubular secretion.[24] On the other hand, no significant effect of a combination of glibenclamid/metformin on single-dose memantine pharmacokinetics was found in another study, even though metformin is a substrate of organic cation transporters.[29] A large proportion of our study population received drugs eliminated via tubular secretion and/or OCT inhibitors, but no interactions were revealed. The classification of OCT inhibitors was mostly based on *in vitro* data, thus, the *in vivo* consequences are difficult to predict. Further studies are required to elucidate the interaction potential between OCT inhibitors and memantine.

In the current study, PXR agonists and P-gp inhibitors showed a moderate increase on memantine CL. The enhancement by PXR agonists might be explained by an increased expression of OCTs, as it has been reported for rifampicin.[19] The enhancement by P-gp inhibitors is somewhat counterintuitive, as a slower elimination would be expected from a diminished transport function. Since P-gp is involved in the drug distribution process, an altered distribution of memantine could be a possible explanation. However, as many P-gp inhibitors are also PXR agonists (e.g., simvastatin, omeprazole), the effects of these comedications could not be clearly distinguished. The involvement of P-gp in memantine CL must therefore be examined in further studies. A limitation of these covariate analyses is the classification of the drugs mainly based on *in vitro* data.

It has become evident that membrane transporters are important determinants of drug disposition, and genetic variations altering the function or expression of transporters are interesting targets for pharmacogenetic investigations.[30] In this study, we examined the relationship of polymorphisms in genes of renal cation transporters with memantine elimination. Although the selected SNPs were associated *in vitro* and/or *in vivo* with altered transport, pharmacokinetics or pharmacodynamics of OCT substrates,[10,13,31-34] they did not affect memantine CL. Several explanations for the lack of association are conceivable. On one hand, the naturalistic approach of the study, including patients with comorbidities and several comedications, might have masked subtle effects of genetic variations. The *in vivo* impact of cation transporter polymorphisms has most extensively been studied with metformin. Supporting our hypothesis, the effect of genetic variants on metformin pharmacokinetics appears small and the subsequent effects on clinical response are also limited, above all in studies involving patients.[13,14] On the other hand, it is likely that several redundant cation transporters are implicated in the renal elimination of memantine. A diminished activity of one transporter might therefore be compensated by the other ones. Finally, considerable intersubject differences in OCT expression have been reported suggesting that other mechanisms regulating the expression of renal transporters might be predominant.[13]

Recently, attention has been drawn on genetic variations in nuclear receptors, implicated in the control of drug metabolizing enzyme and transporter expression. In this study, we investigated several polymorphisms in the following nuclear receptors known to be involved in cation transporter expression: PXR, RXRα, PPARγ and CAR.[15-18] A significant relationship between memantine CL with the SNP *NR1I2* rs1513023 was found, with a 16% decreased CL in patients carrying at least one T allele. *In vitro*, this SNP was associated with decreased hepatic CYP3A4 expression.[20] Expecting the same effect, the reduced CL would be a result of lower transporter expression in the kidneys. For further insights into the mechanistic basis underlying the alteration in memantine pharmacokinetics, *in vitro* studies on renal transporter expression related to *NR1I2* rs1513023 genotypes are required.

To date, no prospective studies have been conducted to establish a therapeutic range of memantine for efficacy and tolerability optimization. However, in recently published consensus guidelines, a therapeutic range of 90-150 ng/ml was proposed.[23] In patients, receiving the recommended dose of 20 mg memantine daily and reference alleles for *NR1I2*, the predicted C_{av} levels were in good agreement with the therapeutic range. The proportion of patients exceeding the therapeutic range increased considerably in carriers of the *NR1I2* rs1513023 T allele and/or in case of renal insufficiency with a CL_{creat} of 30 ml/min. According to the prescription information,[2] half of the normal daily dose is recommended for patients with severe renal impairment. The simulations predicted a C_{av} inferior to the therapeutic range for the majority of patients with a Cl_{creat} of 30 ml/min and *NR1I2* reference alleles. On the other hand, a dose of 15 mg per day leaded to similar C_{av} than a 20 mg daily dose in patients with normal renal function and might thus be more appropriate. In accordance with the proposed therapeutic range, patients with memantine C_{av} levels above 150 ng/ml had a significant increased occurrence of AEs. Thus, a dose reduction might be useful if the threshold of 150 ng/ml is exceeded, especially in case of AEs that might limit adherence to the treatment. Due to the cross-sectional study design, AEs were recorded at inclusion when most participants were already receiving memantine for several months. Therefore, it was difficult to differentiate between memantine related AEs and effects of concomitant diseases or medication, and confirmation of the result in other study cohorts is required. With respect to a possible benefit of individual memantine dose-adaption, further studies on the concentration-effect relationship are necessary to establish an evidence-based therapeutic range.

In conclusion, the pharmacokinetic parameters of memantine were adequately described by our population model, showing a predominant role of renal function and gender on memantine CL. To date, this is the first study that comprehensively evaluated the effect of polymorphisms in renal transporters and nuclear receptors on memantine pharmacokinetics. No significant relationship between genetic variations in renal cation transporter genes and memantine CL was found. In contrast, the polymorphism *NR1I2* rs1523130, that potentially alter the expression of transporters in the kidney, was significantly related to slower memantine elimination. However, replication of these results in another group of patients is necessary. These findings are an important contribution to the understanding of interindividual variability in memantine pharmacokinetics and might be useful to improve clinical outcome and tolerability of the treatment.

MATERIALS AND METHODS

Study Population

A total of 108 subjects receiving memantine for at least one month at stable dose were included in this cross-sectional study. Patients were recruited at four hospitals in Switzerland (Lausanne, Geneva, Marsens, Aigle) and in affiliated nursing homes. The sample consisted of 94% inpatients and 6% outpatients. One blood sampling was performed to measure the memantine plasma level, to evaluate the renal function (standard laboratory tests of creatinine, urea), and to extract DNA for the genetic analyses. The presence of adverse events, reported by the patients, doctors and/or nurses, was registered. Additionally, concomitant diseases, comedication, consumption of grapefruit, alcohol and tobacco were recorded.

The study was approved by the local ethics committees (Lausanne University Hospital, Geneva University Hospital, Cantonal Ethic Committee Fribourg) and conducted according to the Good Clinical Practices. Written informed consent, including consent for genetic analysis, was obtained from all patients or their legal representative.

Drug concentration measurement

Blood samples were collected on average 12 hours after medication intake (range: 1-25 hours) in EDTA blood tubes. After centrifugation, the plasma was stored at -20°C until analysis. Memantine plasma levels were determined by high performance liquid chromatography coupled with mass spectrometry, as previously described.[35]

Genotyping

Genomic DNA was extracted from EDTA blood samples with the FlexiGene DNA extraction kit or the QIAamp DNA Blood Mini Kit (QIAGEN, Hombrechtikon, Switzerland) according to the manufacturer's protocols. The following functional and tagging SNPs were selected for genotyping based on previously published *in vitro* or *in vivo* pharmacogenetic studies and on their frequency in the Caucasian population.[10,13,20,21,31-34,36,37] Genotyping was performed by real-time polymerase chain reaction with 5'-nuclease allelic discrimination assays (ABI PRISM 7000; Applied Biosystems, Rotkreuz, Switzerland) according to manufacturer's instructions or previous studies:[38] *SLC22A1* rs628031 (C__8709275_60), rs72552763 (C__34211613_10), rs622342 (C__928527_20), rs34130495 (Custom TaqMan SNP Genotyping Assay), rs34059508 (C__30634080_20), rs12208357 (C__30634096_10), *SLC22A2* rs2279463 (C__8703416), rs316003 (C__928498_30), rs316019 (C__3111809_20), rs624249 (Custom TaqMan SNP Genotyping Assay); SLC22A5 rs2631367 (C__26479161_30); *SLC47A1* rs2289669 (C__15882280_10); *ABCB1* rs2032582, *ABCB1* rs1045642, *ABCB1* rs1128503 (C__7586662_10), *ABCB1* rs2229109 (C__15951365_20); *NR1I2* rs1523130 (C__9152783_20), rs2472677 (C__26079845_10), rs7643645, (C__1834250_10), *NR1I3* rs2307424, rs4073054 (C__25741543_10), rs2502815, *RXRA* rs3132297 (C__9199894_10), rs3818740

(C__27521184_10), *PPARGC1A* rs8192678 (C__1643192_20), *PPARG* rs1801282 (C__1129864_10), rs3856806 (C__11922961_30).

Model-based pharmacokinetic analysis

Structural model. The pharmacokinetic analysis was performed using NONMEM (version *7.1*) [39] with the PsN-Toolkit (version 3.2.4).[40]

A stepwise procedure was employed to identify the model that fitted the data the best, comparing one- vs. multi-compartment models with several absorption types. Memantine CL and V were estimated during the analysis, while k_a was fixed to the mean of the bootstrap resampling analysis, because of the limited data collected during the absorption phase. Since memantine was only administered orally, the estimated pharmacokinetic parameters represent apparent values.

Exponential errors following a log-normal distribution were used to describe interpatient variability of the pharmacokinetic parameters. Proportional, additive and mixed error models were compared to describe the intrapatient (residual) variability.

Covariate model. Covariates potentially influencing the kinetic parameters were incorporated sequentially in the model. The typical value of a given parameter *θ* was modeled to depend linearly on the non-genetic covariate *X*, as shown by the equation:

$$\theta=\theta_0 \cdot (1+\theta_1 \cdot X) \qquad \text{Eq 1}$$

where θ_0 is the mean estimate and θ_1 is the relative deviation of the mean due to the *X* covariate. Age and CL_{creat} were included in the model as relative deviation respectively from the population mean and the limit value of normal renal function in case of CL_{creat}, i.e., 80 ml/min and categorical variables were coded as 0 and 1. CL_{creat} was calculated based on the MDRD formula.[26] In order to characterize the impact of genetic polymorphisms on memantine CL, patients were categorized into genotypic groups as well as according to the number of functional alleles. Activity scores of 2, 1 and 0 were assigned respectively to the fully functional reference (Hom-Ref), heterozygous (Het-LOF), and homozygous (Hom-LOF) diminished/loss of function alleles.[41] CL values were then estimated for each genotype (rich model) and/or for further regrouped (reduced model) sub-populations using the equation:

$$CL=\sum_i CL_i I_i \qquad \text{Eq 2}$$

where CL_i is the typical value for the *i*th genetic group and I_i is an indicator variable taking the value of 1 if an individual carries the *i*th genotype, 0 otherwise. Reduced models, in which the same genotyping group was assigned to Het-LOF and Hom-LOF, as well as those relating CL with the activity score, were tested and compared to the rich model. Competitive models evaluated in this study were linear, power, square root and square equations, as follows:

$$CL=CL_0+\theta_1 \cdot n \qquad \text{Eq 3}$$

$$CL=CL_0 \cdot \theta_1^n \qquad \text{Eq 4}$$

$$CL=CL_0+\theta_1\cdot\sqrt{n} \qquad \text{Eq 5}$$

$$CL=CL_0+\theta_1\cdot n^2 \qquad \text{Eq 6}$$

where n = 0, 1 or 2 is the activity score and θ_1 is the average contribution per allele above that of the Hom-Ref CL (CL_0).

At the end of the univariate analysis, all patient characteristics that showed an influence on the pharmacokinetic parameters were evaluated again by comparing a model containing all the significant covariates with a model from which each factor was deleted sequentially.

Parameter estimation and model selection. The data were fitted by use of the first-order conditional method. As a goodness-of-fit statistics, NONMEM uses an objective function value (OF), which corresponds approximately to minus twice the maximum logarithm of the likelihood of the data. The ΔOF between two models approximates a χ^2 distribution and, along with diagnostic goodness-of-fit plots, is used for models comparison. In the model-building step, a decrease of the OF is considered statistically significant ($p<0.05$) if it exceeds 3.8 for 1 additional parameter. An increase of 5.9 points in the OF ($p<0.01$) was used to test for covariate significance during the backward deletion procedure.

Model validation and Simulation. The bootstrap method with replacement was used to assess the stability of the final model and to construct the confidence intervals (CIs) of the pharmacokinetic parameters using PsN-Toolkit (Version 3.2.4).[40] Five hundred data sets were reconstructed by re-sampling from the original data. The mean values of the parameters obtained were compared with those estimated from the original data. The 95% CIs were calculated for each parameter. In addition, simulations based on the final pharmacokinetic estimates were performed with NONMEM using 1,000 individuals in order to calculate 95% prediction intervals and the C_{av} for individuals with different *NR1I2* rs1523130 genotypes and renal function (CL_{creat} 30 or 80 ml/min) after the administration of 10 mg, 15 mg and 20 mg daily. The concentrations including the 5th and 95th percentiles at each time point were retrieved to construct the prediction intervals. The figures were generated with GraphPad Prism (Version 5.0 for Windows, GraphPad Software, San Diego California USA).

Concentration-side effect analysis

The relationship between log-transformed individual estimates of memantine C_{av} and $AUC_{0-\tau}$ and AEs (constipation, dizziness, headache, somnolence) was investigated by logistic regression analyses. $AUC_{0-\tau}$ was obtained using Dose/CL and C_{av} derived as $AUC_{0-\tau}/\tau$, with τ = 12 or 24 h. The association between different memantine C_{av} cut-off levels (100 ng/ml, 150 ng/ml, 200 ng/ml) and the occurrence of AEs was assessed with chi-square tests. The analyses were performed on STATA software (Version 11.2, StataCorp, College Station, Texas, USA) and a p-value ≤0.05 was considered statistically significant.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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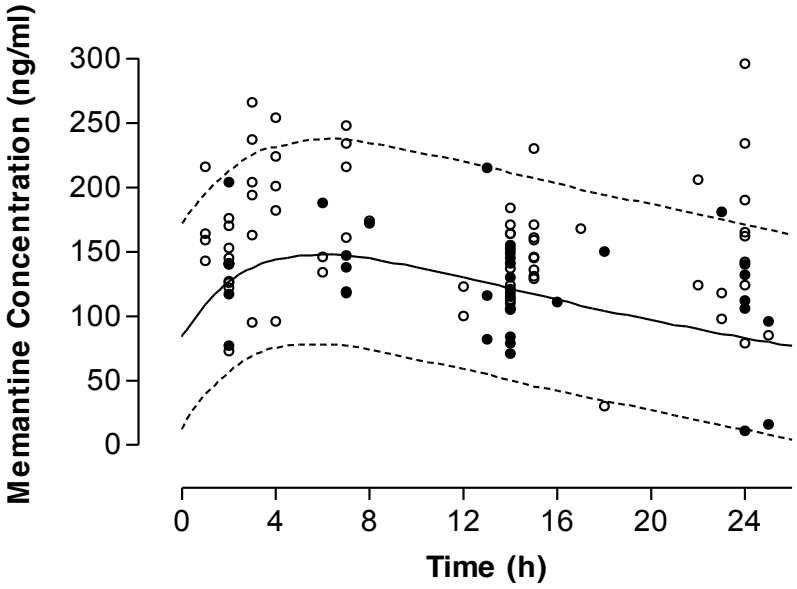

Figure 1: Memantine dose-normalized plasma level-time plots of male (black circles) and female (white circles) patients. Simulations were performed for male patients carrying the *NR1I2* rs1523130 CC genotype and receiving 20 mg of memantine per day (solid line represents average population prediction value; dashed lines, 95% prediction intervals).

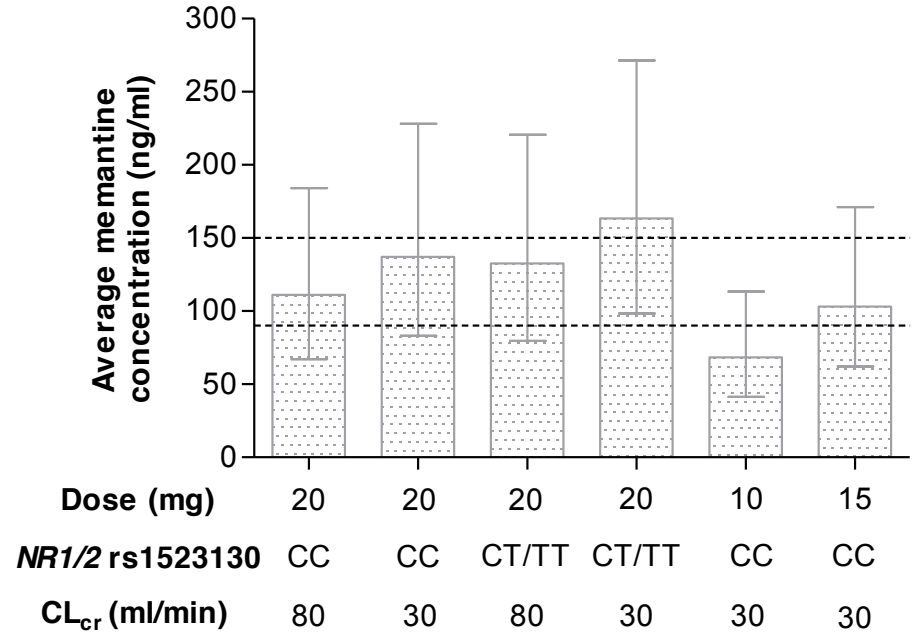

Figure 2: Predicted average concentrations (bars) with 95% prediction interval for male patients with various *NR1I2* rs1523130 genotypes and renal function (CL_{creat} = 80 or 30 ml/min) administered 20 mg, 15 mg or 10 mg memantine per day. The suggested therapeutic range between 90 and 150 ng/ml is indicated.[23]

Table 1: Characteristics of 108 model-building patients evaluated in the population pharmacokinetic analysis of memantine.

Baseline characteristic	Model-building patients	
	Value	% or range
Demographic characteristics		
Sex (men/women) (no.)	34/74	31/69
Median age (yr)	83	59-100
Ethnicity (no. of patients)		
Caucasian	106	98
Asian	2	2
Environmental characteristics		
Drug formulation (tablet/solution)	101/7	94/6
Alcohol Consumption (normal/high/no information)	104/2/2	96/2/2
Grapefruit consumption (yes/no)	1/107	1/99
Smoking Status (yes/no)	3/105	3/97
Physiologic characteristics		
CL_{creat} [a] (ml/min)	63	27-147
Creatinine (µmol/l)	90	37-168
Urea (mmol/l)	7.8	3.3-45.8
Concomitant medications (no/yes)		
OCT inhibitors	75/33	69/31
Drug eliminated via tubular secretion	38/70	35/65
PXR agonists	78/30	72/28
P-gp inhibitors	49/59	45/55
P-gp inductors	94/14	87/13

[a] CL_{creat}, creatinine clearance estimated by glomerular filtration rate.[26]

Table 2: Summary of the models used to examine the influence of patient covariates on memantine oral CL.

Non-genetic covariates	Model	θ_0	θ_1	θ_2	ΔOF^c	P
Individual characteristics						
Does SEX influence CL? (male: SEX=1; female: SEX=2)	$\theta_0 (1+ \theta_1 \cdot (SEX-1))$	6.30	-0.22		-11.7	< 0.01
Does AGE influence CL?	$\theta_0 (1+ \theta_1 \cdot AGE^b)$	5.34	-1.12		-9.3	< 0.01
Does ethnicity influence CL? (Caucasian: q_1=1; q_2=0 Asian : q_1=0; q_2=1)	$\theta_0 \cdot q_1 + \theta_1 \cdot q_2$	5.21	7.15		-1.0	NS
Does memantine formulation influence CL? (tablet: FORM=1; solution: FORM=2)	$\theta_0 (1+ \theta_1 \cdot (FORM-1))$	5.29	-0.12		-0.7	NS
Does CL_{creat}[a] influence CL?	$\theta_0 (1+ \theta_1 \cdot CL_{creat}^b)$	5.65	-0.34		-7.3	< 0.01
Does Urea influence CL?	$\theta_0 (1+ \theta_1 \cdot UREA)$	5.43	$5 \cdot 10^{-3}$		0.5	NS
Does alcohol consumption influence CL? (Normal/no info: q_1=1; q_2=0 high : q_1=0; q_2=1)	$\theta_0 \cdot q_1 + \theta_1 \cdot q_2$	5.24	6.02		-2.8	NS
Does grapefruit consumption influence CL? (NO : q_1=1; q_2=0 YES: q_1=0; q_2=1)	$\theta_0 \cdot q_1 + \theta_1 \cdot q_2$	5.27	3.45		-2.2	NS
Does smoking influence CL? (NO : q_1=1; q_2=0 YES: q_1=0; q_2=1)	$\theta_0 \cdot q_1 + \theta_1 \cdot q_2$	5.20	6.44		-0.9	NS
Concomitant medications on CL (DRUG=1 if administered)	$\theta_0 (1+ \theta_1 \cdot DRUG)$					
OCT Inhibitors		5.20	0.03		-0.1	NS
Drug eliminated via tubular secretion		5.11	0.04		-0.3	NS
PXR agonists		5.00	0.18		-5.0	0.02
P-gp Inhibitors		4.67	0.23		-11.0	< 0.01
P-gp Inductors		5.24	$5 \cdot 10^{-3}$		0.0	NS
Genetic covariates	**Model**	θ_0	θ_1	θ_2	ΔOF^c	P
SLC22A2 rs316019 Rich model (Eq 2): I_0: TT; I_1: GT; I_2: GG	$\theta_0 I_0 + \theta_1 I_1 + \theta_2 I_2$	6.94	5.63	5.11	-2.0	NS
SLC22A2 rs316003 Rich model (Eq 2): I_0: CC; I_1: TC; I_2: TT	$\theta_0 I_0 + \theta_1 I_1 + \theta_2 I_2$	6.55	5.17	5.12	-3.1	NS
SLC22A2 rs2279463 Rich model (Eq 2): I_0: GG; I_1: AG; I_2: AA	$\theta_0 I_0 + \theta_1 I_1 + \theta_2 I_2$	6.20	5.30	5.21	-0.30	NS
SLC22A2 rs624249 Rich model (Eq 2): I_0: AA; I_1: CA; I_2: CC	$\theta_0 I_0 + \theta_1 I_1 + \theta_2 I_2$	5.32	4.99	5.58	-2.4	NS
SLC22A1 rs628031 Rich model (Eq 2): I_0: AA; I_1: GA; I_2: GG	$\theta_0 I_0 + \theta_1 I_1 + \theta_2 I_2$	5.64	5.30	5.03	-1.4	NS
SLC22A1 rs72552763 Rich model (Eq 2): I_0: deldel; I_1:GATdel; I_2:GATGAT	$\theta_0 I_0 + \theta_1 I_1 + \theta_2 I_2$	4.60	5.29	5.22	-0.2	NS
SLC22A1 rs622342 Rich model (Eq 2): I_0: CC; I_1: AC; I_2: AA	$\theta_0 I_0 + \theta_1 I_1 + \theta_2 I_2$	6.19	5.09	5.11	-3.6	NS
SLC22A1 rs34059508 Rich model (Eq 2): I_1:GA; I_2: GG	$\theta_1 I_1 + \theta_2 I_2$		4.72	5.25	-0.3	NS

SLC22A1 rs12208357	$\theta_1 I_1+\theta_2 I_2$		5.70	5.16	-1.0	NS
Rich model (Eq 2): I_1: CT; I_2: CC						
SLC22A1 rs34130495	$\theta_1 I_1+\theta_2 I_2$		4.54	5.26	-0.6	NS
Rich model (Eq 2): I_1:GA; I_2: GG						
SLC22A5 rs2631367	$\theta_0 I_0+\theta_1 I_1+\theta_2 I_2$	4.97	5.21	5.61	-2.0	NS
Rich model (Eq 2): I_0:GG; I_1:CG; I_2: CC						
SLC47A1 rs2289669	$\theta_0 I_0+\theta_1 I_1+\theta_2 I_2$	5.26	5.39	4.98	-1.2	NS
Rich model (Eq 2): I_0:AA; I_1: GA; I_2: GG						
ABCB1 3435CT	$\theta_0 I_0+\theta_1 I_1+\theta_2 I_2$	5.27	5.12	5.39	-0.4	NS
Rich model (Eq 2): I_0:TT; I_1: CT; I_2: CC						
ABCB1 2677GT	$\theta_0 I_0+\theta_1 I_1+\theta_2 I_2$	4.97	5.23	5.39	-0.8	NS
Rich model (Eq 2): I_0: TT; I_1:GT; I_2: GG						
ABCB1 1236CT	$\theta_0 I_0+\theta_1 I_1+\theta_2 I_2$	5.17	5.16	5.40	-0.4	NS
Rich model (Eq 2): I_0:TT; I_1: CT; I_2: CC						
ABCB1 1199GA	$\theta_0 I_0+\theta_1 I_1+\theta_2 I_2$	3.71	5.05	5.27	-1.0	NS
Rich model (Eq 2): I_0: AA; I_1: GA; I_2: GG						
NR1I2 rs1523130	$\theta_0 I_0+\theta_1 I_1+\theta_2 I_2$	4.74	5.04	5.87	-6.3	0.04
Rich model (Eq 2): I_0:TT; I_1:CT, I_2: CC						
Recessive model (Eq 2): q1=0 for CT/TT, q1=1 for CC	$\theta_0 (1+ \theta_1 \cdot q_1)$	5.87		-0.16	-5.8	0.02
Linear model [d] (Eq 3)	$\theta_0+\theta_1 \cdot n$	4.59	0.57		-5.6	0.02
Power model [d] (Eq 4)	$\theta_0 \cdot \theta_1^n$	4.59	1.12		-5.7	0.02
Square root model [d] (Eq 5)	$\theta_0+\theta_1 \cdot \sqrt{n}$	4.59	0.68		-4.0	0.04
Square model [d] (Eq 6)	$\theta_0+\theta_1 \cdot n^2$	4.75	0.28		-6.3	0.01
NR1I2 rs2472677	$\theta_0 I_0+\theta_1 I_1+\theta_2 I_2$	5.15	5.34	5.16	-0.3	NS
Rich model (Eq 2): I_0: CC; I_1 TC; I_2: TT						
NR1I2 rs7643645	$\theta_0 I_0+\theta_1 I_1+\theta_2 I_2$	5.77	5.32	5.03	-2.0	NS
Rich model (Eq 2): I_0: GG; I_1 AG; I_2: AA						
NR1I3 rs2307424	$\theta_0 I_0+\theta_1 I_1+\theta_2 I_2$	4.60	5.31	5.19	-1.6	NS
Rich model (Eq 2): I_0: TT; I_1 CT; I_2: CC						
NR1I3 rs4073054	$\theta_0 I_0+\theta_1 I_1+\theta_2 I_2$	4.82	5.41	5.10	-1.6	NS
Rich model (Eq 2): I_0: GG; I_1 TG; I_2: TT						
NR1I3 rs2502815	$\theta_0 I_0+\theta_1 I_1+\theta_2 I_2$	4.23	5.25	5.27	-1.0	NS
Rich model (Eq 2): I_0: TT; I_1 CT; I_2: CC						
RXRA rs3132297	$\theta_0 I_0+\theta_1 I_1+\theta_2 I_2$	5.46	4.70	5.41	-3.2	NS
Rich model (Eq 2): I_0: TT; I_1 CT; I_2: CC						
RXRA rs3818740	$\theta_0 I_0+\theta_1 I_1+\theta_2 I_2$	4.78	5.09	5.45	-1.8	NS
Rich model (Eq 2): I_0: CC; I_1 TC; I_2: TT						
PPARGC1B rs8192678	$\theta_0 I_0+\theta_1 I_1+\theta_2 I_2$	4.85	5.36	5.20	-0.9	NS
Rich model (Eq 2): I_0: TT; I_1 CT; I_2: CC						
PPARG rs1801282	$\theta_0 I_0+\theta_1 I_1+\theta_2 I_2$	6.25	5.39	5.20	-0.7	NS
Rich model (Eq 2): I_0: GG; I_1 CG; I_2: CC						
PPARG rs3856806	$\theta_0 I_0+\theta_1 I_1+\theta_2 I_2$	5.32	5.31	5.22	-0.7	NS
Rich model (Eq 2): I_0: TT; I_1 CT; I_2: CC						

[a] CL_{creat}, creatinine clearance estimated by glomerular filtration rate,[26]

[b] Age and CL_{creat} are expressed as the relative deviation of the individual age and CL_{creat} from the population mean and normal renal function limit value (80 ml/min).

[c] Differences in the NONMEM objective function (ΔOF) compared to the basic structural model, without covariates and with parameters estimates of CL of 5.2 l/h, a V of 172.8 l and a k_a of 0.3 h^{-1}

[d] n =0, 1 or 2, number of functional alleles respectively for the TT, CT and CC patients.

Table 3: Genotype frequencies in the study population (n=108).

Genetic Polymorphisms		Model-building patients		
		Genotypes	Value	Frequency (%)
SLC22A2	rs316019 (A270S)	GG/GT/TT	88/18/2	81/17/2
	rs316003	TT/TC/CC	66/35/7	61/32/6
	rs2279463	AA/AG/GG	84/23/1	78/21/1
SLC22A1	rs624249	CC/CA/AA	38/53/17	35/49/16
	rs628031 (M408V)	GG/GA/AA	39/53/16	36/48/15
	rs72552763 (M420del)	GATGAT/GATdel/deldel	72/35/1	67/32/1
	rs34059508 (G465R)	GG/GA/AA	104/4/0	96/4/0
	rs12208357 (R61C)	CC/CT/TT	95/13/0	88/12/0
	rs34130495 (G401S)	GG/GA/AA	105/3/0	97/3/0
	rs622342	AA/AC/CC	46/48/14	43/44/13
	Number of active alleles[a]	2 active/1 active/0 active	58/49/1	54/45/1
SLC22A5	rs2631367	CC/CG/GG	31/48/29	29/44/27
SLC47A1	rs2289669	GG/GA/AA	34/54/20	31/50/19
ABCB1	rs2032582 (3435C>T)	CC/CT/TT	29/46/33	27/43/30
	rs1045642 (2677G>T)	GG/GT/TT	38/51/19	35/47/18
	rs1128503 (1236C>T)	CC/CT/TT	38/47/23	35/44/21
NR1I2	rs2229109 (1199G>A)	GG/GA/AA	102/5/1	94/5/1
	rs1523130	CC/CT/TT	36/54/18	33/50/17
	rs2472677	TT/TC/CC	42/45/21	39/42/19
NR1I3	rs7643645	AA/AG/GG	54/40/14	50/37/13
	rs2307424	CC/CT/TT	42/54/12	39/50/11
	rs4073054	TT/TG/GG	34/62/12	32/57/11
RXRA	rs2502815	CC/CT/TT	62/44/2	57/41/2
	rs3132297	CC/CT/TT	81/24/3	75/22/3
PPARGC1B	rs3818740	TT/TC/CC	53/45/10	49/42/9
PPARG	rs8192678	CC/CT/TT	38/57/13	35/53/12
	rs1801282	CC/CG/GG	84/22/2	78/20/2
	rs3856806	CC/CT/TT	81/26/1	75/24/1

[a] Active alleles were defined by the absence of one or more of the following amino acid substitutions in *SLC22A1*, according to literature:[31] M408V, M420del, G465R, R61C, G401S.

Table 4: Final population pharmacokinetic parameter estimates of memantine and their bootstrap evaluations (CL/F, mean apparent clearance for male wild-type *NR1I2* rs1523130 patients with normal renal function; V/F, mean apparent volume of distribution; k_a, mean absorption rate constant).

Parameter	Population mean				Bootstrap evaluation			
	Estimate	SE[a] (%)	IIV[b] (%)	SE[c] (%)	Estimate	SE[a] (%)	IIV[b] (%)	SE[c] (%)
CL/F (l/h)	7.51	8	22	57	7.57	9	21	61
$\theta^{d}_{\text{NR1I2 rs1523130}}$	-0.16	30			-0.16	34		
$\theta^{e}_{CL_{creat}}$	-0.30	40			-0.29	43		
θ_{female}	-0.20	26			-0.21	27		
V/F (l)	194	30			242	72		
k_a (h^{-1})	0.30	--			0.30			
σ^{f}	25	19			25	21		

[a] Standard errors of the estimates (SE) are defined as SE/estimate and are expressed as percentages.

[b] Interindividual variability defined as CVs (%).

[c] Standard errors of the coefficient of variations, calculated as (SE/estimate) ^ 0.5, are expressed as percentages.

[d] Relative deviation of CL/F due to *NR1I2 rs1523130* individuals carrying of at least one T allele (Eq 1).

[e] Relative deviation of CL/F as a function of CL_{creat}, estimated using CL/F * (1+ $\theta_{CL_{creat}}$ * (80-CL_{creat})/80)), where 80ml/min is the limit value of normal renal function

[f] Residual additive error, expressed as standard deviation (ng/ml).

Supplementary Table 1: Classification of patients' comedication

Classification	Drugs	Ref
OCT inhibitors	bisoprolol, carvedilol, diclofenac, fluoxetine, ibuprofen, metformin, metoprolol, *O*-desmethyl-tramadol, paroxetine, propanolol, quinine, ranitidine, spironolactone, trimethoprim	1-6
Drugs eliminated by tubular secretion	acetyl salicylic acid, bisoprolol, candesartan, captopril, digoxine, furosemide, hydrochlorothiazide, ibuprofen, levetiracetam, lisinopril, morphine, metformine, methothrexate, quinine, trimethoprim	7-12
PXR agonists	amoxicillin, atorvastatin, carbamazepine, fluvastatine, ginkgo biloba, isradipine, nifedipine, omeprazole, simvastatin, spironolactone, valproic acid	13
P-gp inhibitors	atorvastatin, bisoprolol, candesartan, carvedilol, diltiazem, felodipine, fluoxetine, haloperidol, irbesartan, nifedipine, omeprazol, pantoprazol, paroxetine, propanolol, sertraline, simvastatin, spironolactone	14-16
P-gp inducers	budenoside, morphine, trazodone, valproic acid, venlafaxine	14

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4.3 Article VI: Relationship of *CYP2D6*, *CYP3A5*, *POR* and *ABCB1* genotypes with galantamine plasma concentrations

Summary

In this article, the results of the pharmacogenetic study in patients treated with galantamine are described. Galantamine is extensively metabolized by the enzymes CYP2D6 and CYP3A and is a substrate of the P-glycoprotein. The relationship of genetic variants influencing the activity of these enzymes and transporter with galantamine steady-state plasma concentrations was investigated in 27 patients treated with a constant dose of galantamine. The patients were genotyped for common polymorphisms in *CYP2D6*, *CYP3A4/5*, *POR* and *ABCB1*. The *CYP2D6* genotype appeared to be an important determinant of galantamine pharmacokinetics, with CYP2D6 poor metabolizers presenting 60% higher galantamine plasma concentrations than CYP2D6 extensive metabolizers. Moreover, the results suggest a potential influence of the *ABCB1* 2677G>T polymorphism on galantamine pharmacokinetics. Genetic variations, primarily in *CYP2D6*, might therefore be interesting for further prospective studies, investigating inter-individual variability in clinical outcome and tolerability of galantamine treatment.

Short communication in preparation

Short Communication

RELATIONSHIP OF *CYP2D6*, *CYP3A5*, *POR* AND *ABCB1* GENOTYPES WITH GALANTAMINE PLASMA CONCENTRATIONS

Muriel Noetzli[1], Monia Guidi[2], Karsten Ebbing[3], Stephan Eyer[4], Serge Zumbach[5], Panteleimon Giannakopoulos[6], Armin von Gunten[3], Chantal Csajka[2,7], Chin Bin Eap[1,7]

1. Unit of Pharmacogenetics and Clinical Psychopharmacology, Centre for Psychiatric Neuroscience, Department of Psychiatry, University Hospital Lausanne, Prilly-Lausanne, Switzerland.
2. Division of Clinical Pharmacology and Toxicology, Department of Medicine, University Hospital Lausanne, Lausanne, Switzerland.
3. Service of Old Age Psychiatry, Department of Psychiatry, University Hospital Lausanne, Prilly-Lausanne, Switzerland.
4. Geriatric Medicine and Geriatric Rehabilitation, Department of medicine, University Hospital Lausanne, Lausanne, Switzerland.
5. Fribourg Mental Health Network, Department of Psychiatry, Cantonal Hospital Fribourg, Marsens, Switzerland.
6. Divisions of General Psychiatry, Department of Mental Health and Psychiatry, University Hospital Geneva, Geneva, Switzerland.
7. Department of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Boulevard d'Yvoy 20, 1211 Geneva 4, Switzerland.

Author contributions

Study concept and design: C.B. Eap

Genetic and statistical analyses: M. Noetzli

Calculation of through plasma levels: M. Guidi, C. Csajka

Data interpretation: M. Noetzli, C.B. Eap

Drafting of the manuscript: M. Noetzli

Patient recruitment and study supervision: M. Noetzli, K. Ebbing, S. Eyer, S. Zumbach, P. Giannakopoulos, A. von Gunten, C.B. Eap

Critical revision of the manuscript for important intellectual content: all authors

ABSTRACT

Background: The frequently prescribed antidementia drug galantamine is extensively metabolized by the enzymes CYP2D6 and CYP3A and is a substrate of the P-glycoprotein. We aimed to study the relationship of genetic variants influencing the activity of these enzymes and transporter with galantamine steady-state plasma concentrations.

Methods: In this naturalistic cross-sectional study, 27 older patients treated with a constant dose of galantamine were included. The patients were genotyped for common polymorphisms in *CYP2D6*, *CYP3A4/5*, *POR* and *ABCB1*, and galantamine steady state plasma concentrations were determined.

Results: The *CYP2D6* genotype appeared to be an important determinant of galantamine pharmacokinetics, with CYP2D6 poor metabolizers presenting 60% higher dose-adjusted galantamine plasma concentrations than CYP2D6 extensive metabolizers (median 2.9 versus 1.8 ng/ml·mg, p=0.004).

Conclusion: The *CYP2D6* genotype significantly influenced galantamine plasma concentrations and might therefore be interesting for further studies investigating interindividual variability in clinical outcome and tolerability of the treatment.

INTRODUCTION

Alzheimer's disease is the most common form of dementia, characterized by progressive deterioration of cognition and global functioning. It is recognized that a loss of cholinergic activity significantly contributes to the cognitive decline.[1] Galantamine, indicated for the symptomatic treatment of mild to moderate Alzheimer's disease, enhances the cholinergic function trough inhibition of the enzyme acetylcholinesterase and allosteric modulation of nicotinic receptors.[2]

It is widely accepted that genetic variations in drug metabolizing enzymes and transporters contribute to therapeutic failure and adverse drug reactions.[3,4] Galantamine is mainly metabolized by the liver enzymes cytochrome P450 (CYP) 2D6 and CYP3A, which stands for the group of isozymes CYP3A4, CYP3A5 and CYP3A7.[5,6] In addtion, galantamine is presumably a substrate of the membrane transporter P-glycoprotein (P-gp), implicated in drug absorption, distribution and excretion.[7] Thus, genetic variations in these enzymes and transporter might influence galantamine disposition and clinical outcome. Moreover, polymorphisms in the cytochrome P450 oxidoreductase (POR), a protein that transfers electrons to the CYP enzymes, have been shown to alter CYP3A activity and might, therefore, affect galantamine pharmacokinetics as well.[8,9] Phenotypically, four types of CYP2D6 metabolizers can be distinguished: poor (PM), intermediate (IM), extensive (EM) and ultrarapid metabolizers (UM). Underlying genetic variations defining the CYP2D6 phenotypes are well known.[10] In previous studies, a 25% decreased galantamine clearance and an altered metabolite profile has been shown in CYP2D6 PMs compared to EMs.[6,11] To our knowledge, no studies investigated the

impact of polymorphisms in *CYP3A*, *POR* or *ABCB1* (encoding P-gp) genes on galantamine pharmacokinetics.

The aim of this work was to study the effect of genetic variations in *CYP2D6*, *CYP3A4/5*, *POR* and *ABCB1* on galantamine steady-state plasma concentrations in a cohort of 27 dementia patients from a naturalistic therapeutic setting. Moreover, the relationship between galantamine plasma concentrations and recorded side effects was assessed.

METHODS

Study design and participants

In this cross-sectional study, 27 patients (23 inpatients and 4 outpatients) receiving galantamine at a constant dose for at least one week were included. Patients were recruited at four hospitals (Lausanne, Geneva, Marsens, Aigle) and in affiliated nursing homes. One blood sampling was performed to measure galantamine plasma concentration, to evaluate renal and hepatic function (standard clinical laboratory tests), and to extract DNA for the genetic analyses. The presence of adverse events, reported by the patients, doctors and/or nurses, was registered. Additionally, concomitant diseases, comedication, consumption of grapefruit, alcohol and tobacco were recorded.
The study was approved by the local ethics committees (Lausanne University Hospital, Geneva University Hospital, Cantonal Ethic Committee Fribourg) and conducted according to the Good Clinical Practices. Written informed consent, including consent for genetic analysis, was obtained from all patients or their legal representative.

Drug concentration measurement and estimation of trough concentration

Blood samples were collected in EDTA blood tubes. After centrifugation, the plasma was stored at -20°C until analysis. Galantamine plasma concentrations were determined by high performance liquid chromatography coupled with mass spectrometry, as previously described.[12]

In 16 patients trough concentrations were measured, whereas in 11 patients the blood samples were drawn 1-7 hours after drug intake. In these patients, trough concentrations were estimated with NONMEM (Version 7.1) using the equation of a previously published population pharmacokinetic analysis including age and creatinine clearance (CL_{creat}) as covariates.[11] Thus, the influence of these two covariates in our study population was only tested in the subgroup of 16 patients with measured trough concentration. Since linear pharmacokinetics of galantamine has been demonstrated,[13] the trough concentration was corrected for each patient by the daily dose.

Genotyping

Genomic DNA was extracted from EDTA blood samples with the FlexiGene DNA extraction kit (QIAGEN, Hombrechtikon, Switzerland) according to the manufacturer's protocol. The following SNPs were detected by real-time polymerase chain reaction with 5'-nuclease allelic discrimination assays

(ABI PRISM 7000; Applied Biosystems, Rotkreuz, Switzerland) according to manufacturer's protocols and previous studies:[14] *CYP2D6*3* (rs35742686), *CYP2D6*4* (rs3892097), *CYP2D6*6* (rs5030655), *CYP3A4* rs4646437, *CYP3A5*3* (rs776746), *ABCB1* 2677G>T (rs2032582), *ABCB1* 3435C>T (rs1045642). Gene deletion (*CYP2D6**5) and duplication/multiplication (*CYP2D6**xN) were analyzed by TaqMan copy number assay and long PCR, respectively, as previously described.[14] Internal quality control samples of known genotype were included in all analyses.

Statistical Analysis

Group comparisons were performed with the non-parametric Wilcoxon rank sum test for continuous variables and with the Fisher's exact test for categorical variables. Associations between continuous variables were tested with the Spearman rank-order correlation coefficients. All tests were two-sided and a p-value ≤0.05 was considered statistically significant. Analyses were performed using STATA software (version 12.0; StataCorp, College Station, Texas, USA).

RESULTS

Study population and influence of non-genetic factors

A total of 27 Caucasian patients were included in the study, receiving a galantamine dose of 8 mg (n=7), 16 mg (n=12) or 24 mg (n=8). The sample consisted of 13 men and 14 women with a mean (±SD) age of 82±7 (range 67-91) years with mean (±SD) duration of treatment of 3.5 (±2.3) years. No patient had hepatic impairment, whereas 6 patients had a moderate renal insufficiency (CL_{creat} 30-59 ml/min). Two patients were smokers and one patient had elevated alcohol consumption. The subjects received on average (±SD) 6±3 concomitant drugs. One patient was treated with the strong CYP2D6 inhibitor paroxetine and was further considered as CYP2D6 PM. Weak or moderate CYP2D6 and CYP3A inhibitors were prescribed to respectively 19 and 9 patients.[15]

Steady-state median (IQR) trough concentrations were 19 (12-26) ng/ml, 33 (27-38) ng/ml and 41 (23-61) ng/ml for patients receiving 8 mg, 16 mg and 24 mg galantamine per day, respectively, and the dose-adjusted concentrations ranged between 0.66 and 4.0 ng/ml·mg. Age, gender, smoking, impaired renal function and comedication with weak or moderate CYP2D6 and CYP3A inhibitors were not significantly associated with dose-adjusted galantamine concentrations (data not shown).

Influence of genetic factors

Allele and genotype frequencies are in agreement with previous reports in Caucasians (Table 1), with the exception of *CYP2D6*3*.[14,16] For this null allele, a frequency (95% confidence interval) of 11% (2-29) was observed, which is higher than the frequency of approximately 2% normally detected in the white population.[14,16] Consequently, more CYP2D6 PMs than expected were identified in our study population.

The patients were classified according to their predicted CYP2D6 phenotype.[17] Overall, 6 PMs, 9 heterozygous EMs (hetEMs), 11 homozygous EMs (homEMs) and one UM were identified. The CYP2D6 phenotype was significantly related to galantamine dose-adjusted plasma concentrations, with PMs displaying 60% higher plasma concentrations compared to hetEMs and homEMs (median 2.9 vs. 2.0 ng/ml·mg, p=0.025, and 1.8 ng/ml·mg, p=0.004, respectively) (Table 1, Figure 1). Contrary to expectations, the UM had a similar dose-adjusted plasma concentration than EMs.

No significant relationship was found between *CYP3A4* rs4646437, *CYP3A5**3, *POR**28 *ABCB1* 3435C>T and 2677G>T polymorphisms and dose-adjusted galantamine concentrations. However, a tendency towards lower concentrations was observed in carriers of the *ABCB1* 2677TT genotype compared to the GG genotype (median TT 1.8 *versus* GG 2.3 ng/ml·mg, p=0.086) (Figure 2).

Influence of dose and concentration on side effects

Overall, 11 patients reported no AE while 16 patients experienced at least 1 AE. Of these, 6 patients had gastrointestinal problems (nausea, vomiting, diarrhea, abdominal pain, dyspepsia, anorexia, weight loss) and 10 patients CNS symptoms (dizziness, confusion, insomnia, headache, depression). Neither galantamine doses nor trough concentrations nor genotypes were related to the presence of AEs.

Although the difference is not statistical significant, only one (17%) of the CYP PMs received the maximum dose of 24 mg per day compared to 7 (35%) of the EMs. This finding might indicate an intolerance of the higher dosage in PMs and should be tested in larger cohorts.

DISCUSSION

The person-to-person variability in drug response is a major problem in clinical practice leading to therapeutic failure or adverse effects in individuals or subpopulation of patients.[18] The source of the variability is likely to be manifold, but for many drugs evidence exists that genetic variations in drug-metabolizing enzymes and transporters influence the pharmacokinetics and subsequently the clinical outcome.[3,4] Up to now, only few clinical trials addressed the issue of inter-individual variability in galantamine pharmacokinetics. In this cross-sectional study, we investigated the relationship of genetic polymorphisms in *CYP2D6*, *CYP3A4/5*, *POR* and *ABCB1* with galantamine concentrations in 27 dementia patients.

A significant influence of the predicted CYP2D6 phenotype on dose-adjusted galantamine plasma concentrations was observed. CYP2D6 PMs displayed 60% higher concentrations compared to CYP2D6 EMs, while homEMs and hetEMs had similar plasma levels. Even though the influence of the CYP2D6 PM status was more pronounced in our study population, these results are in agreement with a previously published population pharmacokinetic study including 356 patients of two phase III clinical trials genotyped for CYP2D6.[11] Their model indicated a 25% reduced galantamine clearance in CYP2D6 PMs (n=20) and a similar clearance among homEMs and hetEMs.[11] In contrast, in a single

low dose study with 4 mg galantamine, no apparent difference in plasma concentrations was seen between two CYP2D6 EMs and two PMs, but the metabolite profile was substantially different.[6] In EMs, urinary metabolites resulting from O-demethylation by CYP2D6 represented 33% of the dose compared with 5% in PMs, which showed correspondingly higher urinary excretion of unchanged galantamine and its N-oxide, formed by CYP3A.[6] In none of these studies CYP2D6 UMs were taken into account. Since only one UM was detected in our study, it was not possible to draw a conclusion either. According to the FDA drug label information, no dose-adjustment is necessary in PMs as the dose is individually titrated to tolerability.[19] Nevertheless, the knowledge of a patient's *CYP2D6* genotype might be useful to avoid concentration-related AEs in PMs.

A tendency towards lower dose-adjusted galantamine concentrations was observed in carriers of the *ABCB1* 2677TT genotype compared to the GG genotype. It might be that the *ABCB1* 2677G>T polymorphism influence galantamine concentrations through altered absorption, distribution and/or excretion. On the other hand, it has also been shown that individuals homozygous for the *ABCB1* 2677T allele have enhanced constitutive CYP3A4 expression in liver and intestine compared with those homozygous for the G allele.[20] This difference in CYP3A activity might therefore explain the tendency towards lower galantamine concentrations in *ABCB1* 2677TT carriers. The lack of a significant relationship between polymorphisms in *CYP3A4/5*, *POR* and *ABCB1* might be due to the predominant influence of the CYP2D6 phenotype and the small study population and should, therefore, be repeated in other cohorts.

Recently, a therapeutic range of 30-60 ng/ml was proposed for galantamine.[21] In the study population, the trough plasma concentrations corresponded well to the suggested range for the recommended maintenance doses of 16 mg and 24 mg daily. Although a dose related occurrence of AEs has been reported,[22] no relationship between galantamine dose or plasma concentrations and the presence of AEs was found. This result might be a consequence of the cross-sectional study design. Prevalent AEs were recorded at inclusion when most patients were already receiving galantamine for several months. Therefore, it was complicated to differentiate between galantamine related AEs and effects of concomitant diseases or medication. Moreover, transient AEs were no more present and intolerant patients would already have reduced the dose or discontinued the treatment and be ineligible.

In conclusion, the present study shows for the first time in a naturalistic setting the significant influence of the CYP2D6 phenotype on galantamine pharmacokinetics, with CYP2D6 PMs displaying 60% higher dose-adjusted plasma concentrations than CYP2D6 EMs. Moreover, the results suggest a potential influence of the *ABCB1* 2677G>T polymorphism. Since it is of special importance for elderly patients with cognitive impairment to avoid multiple medication changes due to non-response or side effects, an individual galantamine dose-adjustment might be useful. Therefore, prospective studies including a larger number of patients and investigating the clinical effectiveness and tolerability of galantamine with respect to plasma concentrations and genotypes are required.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1: Genotype frequencies in the study population (n=27) and dose-adjusted plasma concentrations in genotypes.

Genotype	Frequency			Dose-adjusted plasma levels (ng/ml•mg)		
	n	(%)	95% CI (%)	Median	Interquartile range	p-value
CYP2D6[a]						
homEM	11	41	22-61	1.8	1.5-2.3	
hetEM	9	33	17-54	2.0	1.0-2.4	0.676[c]
PM	6	22	9-42	2.9	2.5-3.5	0.004[c]
UM	1	3	0-19[b]	2.1		
***CYP3A4* rs4646438**						
CC	22	81	62-94	2.0	1.5-2.5	0.662
CT	5	19	6-38	2.1	1.3-2.3	
***CYP3A5* *3**						
*1/*3	3	11	2-29	2.3	1.0-2.6	1.000
*3/*3	24	89	71-98	2.0	1.5-2.5	
POR*28						
CC	13	48	29-68	1.6	1.3-2.4	0.356
CT	12	45	25-65	2.0	1.9-2.4	
TT	2	7	1-24	2.5	1.8-3.2	
***ABCB1* 3435CT**						
CC	6	22	9-42	2.3	1.5-3.0	0.600[d]
CT	15	56	35-75	2.3	1.5-2.6	
TT	6	22	9-45	1.8	1.0-2.0	
***ABCB1* 2677GT**						
GG	10	37	19-58	2.3	1.5-3.2	0.192[d]
GT	12	44	25-65	2.1	1.5-2.5	
TT	5	19	6-38	1.8	1.0-2.0	

a) CYP2D6: PM**,** poor metabolizer: *4/*4 (n=1), *4/*5 (n=1), *3/*4 (n=3), medication with strong CYP2D6 inhibitor (n=1); hetEM**,** heterozygous extensive metabolizer: *1/*4 (n=7), *1/*5 (n=1); *1/*6 (n=4); homEM, homozygous extensive metabolizer: *1/*1 (n=11), *4/*xN (n=1); UM, ultrarapid metabolizer: *1/*XN or *XN/*XN, (n=1).

b) One-sided, 97.5% confidence interval.

c) P value refers to homozygous extensive metabolizers (homEM) versus heterozygous extensive metabolizers (hetEM) and poor metabolizers (PM), respectively.

d) P values determined by Wilcoxon test; dominant model grouped by the presence of at least one variant allele versus wild type.

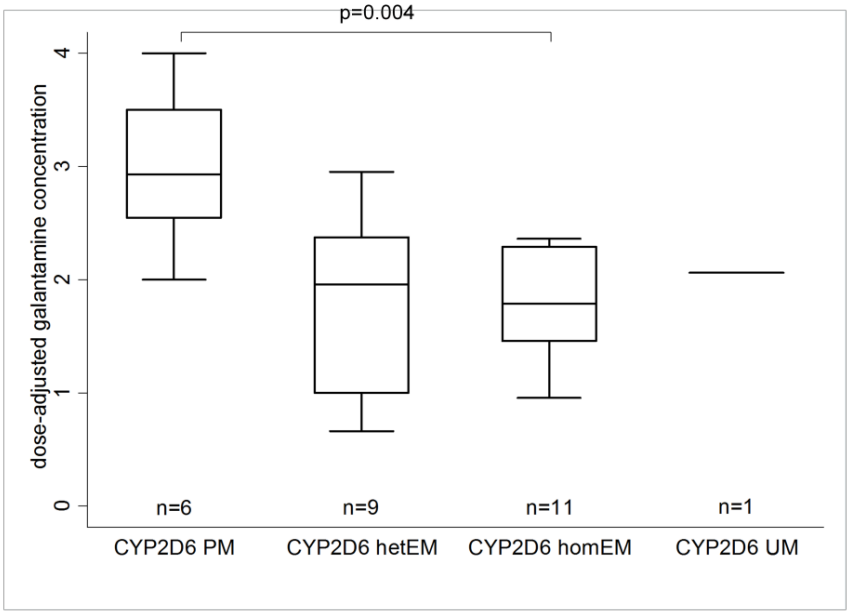

Figure 1: Influence of the predicted CYP2D6 phenotype on dose-adjusted galantamine trough concentrations.

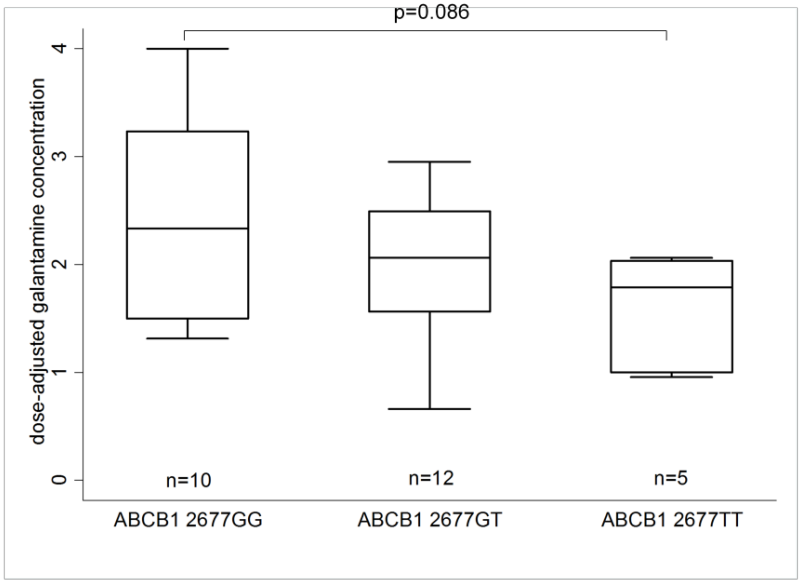

Figure 2: Influence of the ABCB1 2677G>T genotype on dose-adjusted galantamine trough concentrations.

4.4 Rivastigmine

A total of 44 patients treated with rivastigmine were included in the pharmacogenetic study. Patients were administered several different rivastigmine dosages with patch or capsule formulation (Table 1), which resulted in a very heterogeneous dataset.

Table 1: Study participants treated with rivastigmine

	Daily dose	Number of patients	Percentage (%)
Capsule	3 mg	4	9
	4.5 mg	2	5
	6 mg	8	18
	9 mg	3	7
	12 mg	1	2
	Total	***18***	***41***
Patch	4.6 mg	11	25
	9.5 mg	15	34
	Total	***26***	***59***

Rivastigmine plasma concentrations were determined and the previously reported high inter-individual variability was confirmed (Figure 1).[66,67] Despite this observation, no genotyping was performed for pharmacogenetic analyses given that statistical power was lacking for several reasons. First, a comparison of the plasma concentrations by means of normalization by the daily dose was circumvented by the non-linear pharmacokinetics of rivastigmine.[68,69] Therefore, analyses would only be possible in subgroups of patients with the same dosage, which were size limited in our study population. Furthermore, rivastigmine has a short half-life of approximately 1.5 h after capsule administration leading to high fluctuations between peak and trough concentrations.[69] Consequently, estimations of trough plasma concentrations using a general pharmacokinetic equation ($C_{trough}=C_{measured}*e^{-((\ln 2/t1/2)*dt)}$, $t_{1/2}$=half-life, dt=time between medication intake and blood sampling) with half-life reported in the literature are not accurate enough for the purpose of the study. Finally, a larger study sample would be necessary for population pharmacokinetic investigations.

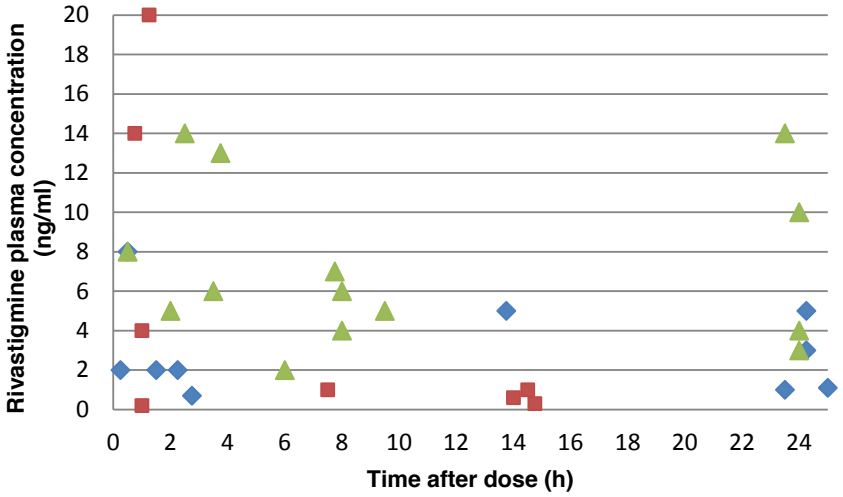

Figure 1: Measured rivastigmine plasma concentrations for patients treated with the following dosages: patch 4.6 mg/24 h (green triangles, n=11), capsules 3 mg b.i.d. (red squares, n=8) and patch 9.5 mg/24 h (blue squares, n=15).

In 57% (n=25) of the patients at least one adverse event, including gastrointestinal problems, dizziness and fatigue, was reported. No significant relationships were found between the occurrence of adverse events and the daily rivastigmine doses. Moreover, analyses were performed in subgroups of patients in which peak or trough concentrations were measured. No associations were revealed between the frequency of adverse events and peak or trough concentrations. In contrast to previous studies, the patch formulation was not related to a lower occurrence of adverse events in our study population. This might be due to the cross-sectional study design, as adverse events were recorded at inclusion, when most patients were already receiving the treatment for several months. Therefore, transient adverse events were no more present and intolerant patients would have already reduced the dose or discontinued the treatment and be ineligible. However, an indication of a better tolerability of the patch might be that only one patient (5.5%) was treated with the highest therapeutic dose of rivastigmine in the capsule group (6 mg b.i.d.) versus 15 patients (58%) in the patch group (9.5 mg/24h). In previous clinical trials, the 9.5 mg/24h patch has been shown to provide comparable rivastigmine exposure to the 6 mg b.i.d capsule administration, with similar efficacy but three times fewer adverse events reports due to the favorable pharmacokinetic profile.[70] Thus, significantly more patients reached the highest dose in the patch group compared to the capsule group, which is in agreement with our finding.[70]

In conclusion, no pharmacogenetic analyses have been performed in the study population receiving rivastigmine due to the heterogeneity of the dataset. However, a large interindividual variability in plasma concentrations was observed. Thus, pharmacogenetic studies in larger study populations with uniform dosage regimens would be of interest. In the present study, preliminary analyses revealed no significant relationships between the occurrence of adverse events and rivastigmine dose, plasma concentrations or formulation.

Chapter 5: Discussion and Perspectives

In this last chapter the results of this work are discussed and some perspectives for future research on the subjects are given.

5.1 Analytical methods

A prerequisite for any pharmacokinetic study and for TDM is a suitable bioanalytical procedure for the measurements of the plasma concentrations of the drugs.
The HPLC-MS method, that was developed and validated for the determination of antidementia drugs in the plasma of the participants of the pharmacogenetic study, is described in Article II. It is the first method published that allows the simultaneous determination of all four antidementia drugs, namely donepezil, galantamine, rivastigmine (and its metabolite NAP 226-90) and memantine. The sample pre-treatment was performed by mixed-mode SPE and the separation of the compounds was achieved by reverse phase chromatography. The method was validated according to the recommendations of international guidelines with satisfactory results. Moreover, the procedure was successfully applied to the 300 study participants, showing to be reliable and robust over the concentration range normally measured in patients.

Since it was planned to introduce the concentration measurements of antidementia drugs in the routine TDM-service of our laboratory, the HPLC-MS procedure was transferred to the newly acquired UPLC-MS/MS system in order to benefit from the numerous advantages of this new technology. The procedure transfer is described in Article III. In the HPLC-MS procedure, the drugs were isolated from 500 µL plasma by SPE, which is a powerful procedure to obtain clean extracts. However, the higher specificity of the UPLC-MS/MS compared to HPLC-MS allows the analysis of less clean extracts with satisfactory results. Therefore, the extraction procedure was simplified and a protein precipitation was used with the advantages of a faster sample preparation and lower costs. In addition, the higher sensitivity of the MS/MS allowed to reduce the amount of required plasma to 250 µL and to decrease the LLOQ of rivastigmine and its metabolite to better correspond to the low concentrations measured in patients. Moreover, the run time was shortened from 15 min to 4.5 min. The UPLC-MS/MS method was fully validated and a method comparison between HPLC-MS and UPLC-MS/MS was performed, showing similar results between the two procedures. During routine use of the method, the reliability will be monitored by internal and external quality control samples. Moreover, attention has to be paid to analytical interferences with comedication, since the elderly population taking antidementia drugs is often polymedicated. However, by the use of the highly specific UPLC-MS/MS technology and of isotope-labeled internal standards compensating for potential signal suppression, the risk of analytical inferences has been minimized.

For a considerable number of psychopharmacologic compounds, the quantification of the medications' plasma concentration has become clinical routine for dose adjustment.[32] Clear evidence of the benefits of TDM has been given for tricyclic antidepressants, numerous antipsychotic drugs and for conventional mood stabilizing drugs.[32] Presently, TDM is rarely used for antidementia drugs, though

there is some evidence that it can be useful since a relationship between plasma concentrations and response to treatment and/or tolerability has been reported.[71-73] The following aspects support the use of TDM for antidementia drugs. First, a large inter-individual variability in plasma concentrations was observed in pervious clinical trials, and could be confirmed by the results of our pharmacogenetic study. As substrates of CYP2D6 and CYP3A metabolic enzymes, donepezil and galantamine pharmacokinetics is affected by environmental and genetic factors influencing the activity of these enzymes. TDM might be beneficial to reveal genetically determined abnormalities in the metabolism, but also to monitor the plasma concentrations when administered in combination with comedication inhibiting or inducing CYP2D6 or CYP3A. Moreover, age-related changes in physiologic functions and comorbidities might affect the pharmacokinetics of antidementia drugs. In particular, a decreased renal function could lead to increased plasma concentrations of memantine. Finally, there is some evidence supporting the utility of antidementia drugs as a chronic treatment that reduces the progression and associated burden of the disease rather than purely to relieve the symptoms.[15] Thus, the detection of non-adherence to therapy through TDM might be beneficial, especially because non-adherence is a particular problem in patients with cognitive deficits.[58]

In the recently published "AGNP Consensus Guidelines for Therapeutic Drug Monitoring in Psychiatry: Update 2011", therapeutic ranges for antidementia drugs are proposed, which are based on pharmacokinetic studies with therapeutically relevant doses.[32] Only for donepezil, the therapeutic range was based on a concentration-response study.[32,71] To recommend a regular monitoring with the available data is not possible, but it can be suggested in special cases such as an insufficient clinical improvement, adverse events, comedication that are inhibitors or inducers of drug metabolizing enzymes or suspected non-adherence.
The data, which will be collected in future in our TDM service, might contribute to evaluate whether a TDM of antidementia drugs could help to minimize the risk of adverse events and to increase the probability of clinical effectiveness. However, prospective concentration-response studies to improve the quality of data on therapeutic reference ranges are required.

5.2 Pharmacogenetics of Donepezil

A total of 129 patients treated with a constant dose of donepezil were included in the pharmacogenetic study on antidementia drugs. The patients were genotyped for polymorphisms in *CYP2D6*, *CYP3A*, *POR*, *NR1I2* and *ABCB1* to investigate their influence on donepezil steady-state concentrations as well as on the occurrence of side effects. The results are reported in Article IV.
The *CYP2D6* genotype appeared to be the major genetic factor influencing donepezil plasma concentrations. Heterozygous extensive metabolizers and poor metabolizers demonstrated significantly higher plasma concentrations than homozygous extensive metabolizers. Moreover, the ultrarapid metabolizers had lower donepezil concentrations than homozygous extensive metabolizers and might, therefore, be prone to non-response to donepezil treatment. Only one previous study examined the relationship between CYP2D6 genotypes and donepezil plasma concentrations.[55] In

contrast to our results, no significant relationship was revealed, which might be due to the limited sample size (n=42) and the lack of poor metabolizers. Nevertheless, their findings showed a tendency towards lower plasma concentrations in homozygous extensive metabolizers compared to heterozygous extensive metabolizers, and in ultrarapid metabolizers compared to extensive metabolizers, which is in line with our results.[55] Further accentuating the importance of CYP2D6 in donepezil metabolism, we measured significantly higher plasma concentrations in patients receiving CYP2D6 inhibitors compared to patients without CYP2D6 inhibiting comedication. In addition, the frequency of subjects experiencing at least one side effect related to donepezil treatment was significantly higher in CYP2D6 poor metabolizers and patients taking strong CYP2D6 inhibitors. This could be due to the higher trough concentrations observed in these subjects, even though the difference did not reach statistical significance. Overall, these findings indicate that a dose-adaption through monitoring of donepezil plasma concentrations could be beneficial to avoid side effects in CYP2D6 poor metabolizers and in patients taking CYP2D6 inhibitors as comedication. However, the confirmation of our results in prospective studies is required.

Interest in the electron transferring enzyme POR and the nuclear receptor PXR resulted from the search on variations in genes influencing CYP3A expression and activity. In this study, a significant association of the *NR1I2* rs1523130 SNP with donepezil concentrations was found. Since *in vitro* studies in liver tissues demonstrated different levels of CYP3A4 expression among *NR1I2* rs1523130 genotypes, this observation could be explained by differences in CYP3A4 activity.[45,47] Because PXR is implicated in the expression of several phase I and phase II drug-metabolizing enzymes as well as transporters including P-pg, the influence of the *NR1I2* rs1523130 might also be mediated by other factors than CYP3A expression. The most common studied *POR* polymorphism is the SNP rs1057868C>T, defining the allele *28. In a previous study, our group showed an influence of the *POR**28 polymorphism on CYP3A activity in two independent cohorts, with *POR**28 TT carriers presenting a 1.6-fold higher CYP3A activity than carriers of the C allele.[31] In line with these findings, we observed lower donepezil plasma concentrations in patients carrying at least one *POR**28 T allele. However, the difference reached statistical significance only in CYP2D6 extensive metabolizers, which suggests a confounding of the effect by the other *CYP2D6* genotypes when considering the whole study population.

The thoughtful assessment of genetic factors that could affect donepezil pharmacokinetics extended the current knowledge on donepezil pharmacogenetics. The data used for the present investigations were obtained from a larger study population than in previous pharmacogenetic trials. Furthermore, this is the first study to examine the relationship of SNPs in *POR* and *NR1I2* with donepezil plasma concentrations. The findings on donepezil presented in this work are based on trough plasma concentrations calculated by means of a pharmacokinetic equation depending on the average half-life observed in patients in previous studies and on the time between medication intake and blood sampling. Because of the long half-life of the drug (70 hours),[74-77] resulting in small differences between peak and trough concentrations, our estimation of the trough concentrations is considered to be suitable for the objectives of the study. However, the influence of genetic and clinical factors on

donepezil pharmacokinetics is presently investigated with a more sophisticated approach. In collaboration with the department of clinical pharmacology at the CHUV (Prof Chantal Csajka, Dr Monia Guidi), a population pharmacokinetic model for donepezil has been established, using the NONMEM software. A manuscript reporting the results of the population pharmacokinetic analysis will be prepared shortly.

Recently, attention was drawn on the SNP rs35599367 in intron 6 of the *CYP3A4* gene, defining the allele *22. *In vitro* this SNP markedly affected the CYP3A4 expression and *in vivo* it modified dose requirements and/or plasma concentrations of CYP3A4-metabolized drugs.[78-80] In view of these findings, we are presently analyzing this polymorphism in our study population to investigate its effect on donepezil pharmacokinetics. Further investigations could include SNPs in other nuclear receptors, such as CAR, influencing the expression of drug metabolizing enzymes and transporters.

5.3 Pharmacogenetics of Memantine

In the pharmacogenetic study, 108 patients receiving memantine at steady-state conditions were included. The patients were genotyped for SNPs in genes of renal cation transporters and nuclear receptors that are involved in transporter expression, and their effect on memantine clearance was assessed. The results of these investigations are described in Article V.

In collaboration with the department of clinical pharmacology at the CHUV, a population pharmacokinetic model was developed, integrating clinical and genetic factors. A dominant influence of the renal function was observed, which is in agreement with the extensive renal elimination of memantine and has previously been shown in a single dose study in elderly volunteers.[81] Moreover, the model demonstrated slower memantine elimination in female patients. In contrary to expectations, no significant relationship was found between genetic variations in renal cation transporters, namely OCTs, OCTN, MATE and P-gp, and memantine pharmacokinetics, although the selected variants were associated *in vitro* and/or *in vivo* with altered transport activity.[82-87] Several explanations are conceivable for the lack of association, including the naturalistic study design that might have masked subtle effects of the genetic variations or the implication of several redundant cation transporters in the renal elimination of memantine compensating for a diminished activity of one of the transporters. Furthermore, since considerable intersubject differences in OCT expression have been reported, mechanisms regulating the expression of renal transporters might be predominant in determining memantine elimination.[85] Thus, we investigated several polymorphisms in genes of the nuclear receptors PXR, CAR, RXRα and PPARγ, which are known to be involved in cation transporter expression.[88-91] The SNP *NR1I2* rs1513023 was significantly related to memantine clearance. As mentioned above, *in vitro* this SNP was associated with differential hepatic CYP3A4 expression among genotypes.[45] A similar effect may be hypothesized for transporter expression in the kidneys, influencing memantine elimination.

The presence of side effects in our study population was analyzed with respect to the proposed therapeutic range for memantine of 90-150 ng/ml.[32] In good agreement, in patients with average

concentrations superior to 150 ng/ml, a significantly increased occurrence of side effects was observed. Thus, a dose reduction might be useful if the threshold of 150 ng/ml is exceeded, especially in case of AEs that might limit adherence to the treatment. Moreover, simulations were performed to predict and compare average plasma concentrations in patients receiving 10 mg, 15mg or 20 mg memantine per day with different *NR1I2* rs1513023 genotypes and normal or impaired renal function. According to the prescription information,[35] half of the normal daily dose, i.e. 10 mg, is recommended for patients with severe renal impairment (creatinine clearance<30 ml/min). Interestingly, the simulations predicted for patients with a creatinine clearance of 30 ml/min and *NR1I2* reference alleles lower average concentrations than observed in patients with normal renal function. Thus, a dose of 15 mg per day might be more adequate, as it leads to similar steady-state plasma concentrations as in patients without renal impairment taking 20 mg memantine per day. It will be interesting to pay attention to this issue in the routine TDM analyses in patients with known renal function.

To our knowledge, this is the first pharmacogenetic study on memantine. Our findings improved the understanding of inter-individual variability in memantine pharmacokinetics and might be useful in the context of individual dose-adaption for a better efficacy and safety of the treatment. However, the replication of the results in other groups of patients is necessary. The domain of transporter pharmacogenetics is presently emerging and, therefore, it is likely that new genetic variants will be described that could be examined regarding memantine pharmacokinetics. Moreover, a better characterization of the spectrum of transporters for which memantine is a substrate would be advantageous for further pharmacogenetic studies. Our analyses on polymorphisms in nuclear receptors were exploratory. *In vitro* studies on renal transporter expression related to genetic variations in nuclear receptors, in particular related to *NR1I2* rs1513023 genotypes, would be required for a profound understanding of the results and for further investigations.

5.4 Pharmacogenetics of Galantamine

In the pharmacogenetic study, 27 patients were included receiving a treatment with galantamine. We investigated the relationship of polymorphisms in *CYP2D6*, *CYP3A*, *POR* and *ABCB1* genes with galantamine concentrations. The results are reported in a short communication (Article VI).

We demonstrated 60% higher galantamine trough concentrations in CYP2D6 poor metabolizers compared to CYP2D6 extensive metabolizers, while homozygous and heterozygous extensive metabolizers had similar plasma levels. Interestingly, more CYP2D6 poor metabolizers than expected were identified in our study population, which allowed to find a statistical significant influence of the *CYP2D6* genotypes. Our findings confirmed the results of a population pharmacokinetic study, showing a 25% reduced galantamine clearance in CYP2D6 poor metabolizers and a similar clearance among homozygous and heterozygous extensive metabolizers.[92] Only one ultrarapid metabolizer was detected in our study population, thus, it was not possible to draw a conclusion for this genotype. According to the FDA drug label information, no dose-adjustment is necessary in poor metabolizers as

the dose is individually titrated to tolerability.[93] Nevertheless, the knowledge of a patient's *CYP2D6* genotype might be useful to avoid concentration-related side effects in poor metabolizers. No significant relationship was found between polymorphisms in *CYP3A*, *POR* and *ABCB1* genes and galantamine concentrations, which might be due to the predominant influence of the *CYP2D6* genotype and the small study population. Therefore, the genetic analyses on *CYP3A*, *POR* and *ABCB1* should be repeated in larger cohorts for sound conclusions.

In the present study, we showed for the first time in a naturalistic setting the significant influence of the *CYP2D6* genotype on galantamine pharmacokinetics. However, the sample size limited the analyses of SNPs potentially influencing galantamine concentrations as well as the investigations on side effects. For further pharmacogenetic studies, a larger cohort is necessary, for which it will also be possible to perform population pharmacokinetic analyses.

5.5 General remarks and perspectives on the pharmacogenetic study on antidementia drugs

The aim of a pharmacological treatment is to administer effective and well tolerated drugs to patients. For most drugs, substantial differences in treatment response exist among patients, which is partly due to inter-individual variabilities in plasma concentrations of the drugs. Accordingly, a standard dose of a drug might not be suitable for every patient. An individual dose adjustment, taking several factors into account that influence the pharmacokinetics of a drug, has been shown to improve efficacy and safety of treatments.

Through the pharmacogenetic study presented in this work, we improved the current understanding of clinical and genetic factors leading to an inter-individual variability in plasma levels of antidementia drugs. Therefore, these findings could contribute, together with the work of other research groups, to a more personalized therapy approach in AD patients treated with acetylcholinesterase inhibitors or memantine, with individual dose adaption according to drug plasma concentrations and/or genotypes. Thanks to the cross-sectional design of the study and large inclusion criteria, the protocol was simple and the participation minimally invasive for the patients. However, the heterogeneity of the population and the different durations of treatment at inclusion limited the assessment of efficacy and safety. Though, our results could be taken into account for the design of prospective studies investigating the clinical impact of the genetic variations, and of other clinical factors influencing the pharmacokinetics of the drugs, on the efficacy and tolerability of the treatment with antidementia drugs. In addition, concentration-response studies are requested to establish evidence based therapeutic ranges of antidementia drugs and to evaluate the usefulness of TDM in these drugs.

Hopefully, increasing the knowledge of pharmacogenetics and of other factors influencing drug response, together with more advanced technologies and decreased laboratory costs for genotyping and drug concentration measurements, will enable us to establish a more personalized therapy approach for AD patients. Through an individual selection of drug regimen and dose, the tolerability of the treatment might be improved and higher therapy success rates might be achieved.

5.6 General Conclusions

Currently, the three acetylcholinesterase inhibitors donepezil, galantamine and rivastigmine and the NMDA-antagonist memantine are licensed for the treatment of AD. Meta-analyses report consistent benefits of these drugs regarding cognition and global assessment, but with small treatment effects.[94-96] Furthermore, it has been shown that discontinuation rates are around 40% and 80% after 12 and 24 months of acetylcholinesterase inhibitor treatment, respectively, with ineffectiveness, intolerance or inconvenient dosing schedule as leading reasons for the discontinuation.[97] At present, no cure for AD is available. Thus, even if an initial improvement is observed in the first months of treatment, the cognitive and behavioral decline cannot be prevented. However, an optimization of the current treatment to maximize the effectiveness and tolerability should be intended, for example through TDM and/or genotyping. The issue of inter-individual variability in pharmacokinetics and pharmacodynamics of antidementia drugs has not been extensively addressed up to now. The pharmacogenetic study presented in this work might, therefore, contribute to a better tailored pharmacotherapy for AD patients, as we demonstrated that genetic factors account for a part of the intersubject variability in pharmacokinetics of donepezil, galantamine and memantine in patients recruited from a naturalistic therapeutic setting. Due to the cross-sectional study design, prevalent side effects were recorded at inclusion when most patients were already receiving the medication for several months. Therefore, it was complicated to differentiate between therapy related side effects and effects of concomitant diseases or medication. Moreover, transient side effects were no more present and intolerant patients would already have reduced the dose or discontinued the treatment and be ineligible. Despite these limitations, significant associations between the predicted CYP2D6 phenotype and side effects related to donepezil treatment, and between the 150 ng/mL plasma concentration threshold and side effects related to memantine treatment were found.

Taken together, these findings suggest a usefulness of TDM and genotyping in the treatment of AD. However, prospective studies investigating the clinical effectiveness and tolerability of antidementia drugs with respect to plasma concentrations and genotypes are required. Moreover, pharmacoeconomic aspects have to be taken into account.

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Appendix

Quantification of nicotine, cotinine, trans-3-hydroxycotinine and varenicline inhuman plasma by a sensitive and specific UPLC–tandem mass-spectrometry procedure for a clinical study on smoking cessation

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Quantification of nicotine, cotinine, *trans*-3′-hydroxycotinine and varenicline in human plasma by a sensitive and specific UPLC–tandem mass-spectrometry procedure for a clinical study on smoking cessation☆

Maria Dobrinas[a], Eva Choong[a], Muriel Noetzli[a], Jacques Cornuz[b], Nicolas Ansermot[a], Chin B. Eap[a,c,*]

[a] Unit of Pharmacogenetics and Clinical Psychopharmacology, Centre for Psychiatric Neuroscience, Department of Psychiatry, CHUV, University of Lausanne, Hospital of Cery, Prilly, Switzerland
[b] Department of Ambulatory Care and Community Medicine, University of Lausanne, Bugnon 44, 1011 Lausanne, Switzerland
[c] School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Boulevard d'Yvoy 20, 1211 Geneva 4, Switzerland

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ABSTRACT

A sensitive and specific ultra performance liquid chromatography–tandem mass spectrometry method for the simultaneous quantification of nicotine, its metabolites cotinine and *trans*-3′-hydroxycotinine and varenicline in human plasma was developed and validated. Sample preparation was realized by solid phase extraction of the target compounds and of the internal standards (nicotine-d4, cotinine-d3, *trans*-3′-hydroxycotinine-d3 and CP-533,633, a structural analog of varenicline) from 0.5 mL of plasma, using a mixed-mode cation exchange support. Chromatographic separations were performed on a hydrophilic interaction liquid chromatography column (HILIC BEH 2.1 × 100 mm, 1.7 μm). A gradient program was used, with a 10 mM ammonium formate buffer pH 3/acetonitrile mobile phase at a flow of 0.4 mL/min. The compounds were detected on a triple quadrupole mass spectrometer, operated with an electrospray interface in positive ionization mode and quantification was performed using multiple reaction monitoring. Matrix effects were quantitatively evaluated with success, with coefficients of variation inferior to 8%. The procedure was fully validated according to Food and Drug Administration guidelines and to Société Française des Sciences et Techniques Pharmaceutiques. The concentration range was 2–500 ng/mL for nicotine, 1–1000 ng/mL for cotinine, 2–1000 ng/mL for *trans*-3′-hydroxycotinine and 1–500 ng/mL for varenicline, according to levels usually measured in plasma. Trueness (86.2–113.6%), repeatability (1.9–12.3%) and intermediate precision (4.4–15.9%) were found to be satisfactory, as well as stability in plasma. The procedure was successfully used to quantify nicotine, its metabolites and varenicline in more than 400 plasma samples from participants in a clinical study on smoking cessation.

1. Introduction

Smoking represents the major avoidable cause of premature deaths, which makes smoking cessation a substantial part of the strategy against tobacco use, providing clear health benefits [1]. However, few people succeed to stop smoking, because of several factors such as nicotine addiction, environmental, personal and social factors [2].

Nicotine is the psychoactive substance in tobacco and it is the major agent responsible for tobacco addiction [2]. Nicotine replacement therapy (NRT) is thus being used for persons who want to quit smoking, providing the user with a dosage of nicotine sufficient to reduce withdrawal symptoms without further reinforcing the behavior of cigarette smoking [3]. Nicotine is metabolized in the liver by the cytochrome P450 2A6 (CYP2A6) to its main metabolites cotinine and *trans*-3′-hydroxycotinine. The ratio of *trans*-3′-hydroxycotinine to cotinine can be used as a phenotypic marker for CYP2A6 activity and for the rate of nicotine metabolism [4], which in turn can reflect the addiction level and the chances to quit smoking [5]. Varenicline is a partial agonist of the δ4ß2 nicotinic acetylcholine receptor and is prescribed as an aid to smoking cessation for its effects of reducing the severity of nicotine withdrawal symptoms and nicotine craving [6].

Studying the pharmacogenetics of these drugs could improve the success rates of smoking cessation programs, allowing to better adapt them to the characteristics of an individual [7]. Thus,

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* Corresponding author at: Unit of Pharmacogenetics and Clinical Psychopharmacology, Centre for Psychiatric Neurosciences, Department of Psychiatry, CHUV, University of Lausanne, Hospital of Cery, 1008 Prilly, Lausanne, Switzerland.
Tel.: +41 21 643 64 38; fax: +41 21 643 64 44.
E-mail address: chin.eap@chuv.ch (C.B. Eap).

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Table 1
MRM parameters and retention times of the analytes and IS.

	$[M+H]^+$	MRM transition (*m/z*)	Cone voltage (eV)	Collision energy (eV)	t_R (min)[a]
Nicotine	163	163→130	30	20	3.32
Nicotine-d4	167	167→134	30	20	3.33
Cotinine	177	177→80	40	25	1.70
Cotinine-d3	180	180→80	35	25	1.71
trans-3′-Hydroxycotinine	193	193→80	40	25	1.69
trans-3′-Hydroxycotinine-d3	196	196→80	35	25	1.70
Varenicline	212	212→169	45	25	3.31
CP-533,633	240	240→197	35	25	3.30

[a] t_R: Retention time.

determining blood levels of these drugs in smokers who are trying to quit allows studying their metabolism and elimination in relation with genetic markers, and could therefore lead to a better understanding of their efficacy and side effects.

Several analytical methods for the quantification of nicotine and/or its metabolites in different biological samples were described, including plasma [8–11], serum [12–14], urine [15–18], breast milk [19,20] and oral fluid/saliva [12,21]. The majority of methods used liquid chromatography (LC) systems coupled with single quadrupole mass spectrometric (MS) detection [10,14] or triple quadrupole mass spectrometry (MS/MS) [8,9,15,16,19]. Sample preparation was mostly performed by solid phase extraction (SPE) [8–10,12,15,16] or by liquid–liquid extraction (LLE) [13,19]. Several methods have been recently published for the quantification of nicotine and metabolites using hydrophilic interaction liquid chromatography (HILIC) [22–24], which has been demonstrated to be a useful technique for the retention of polar compounds [25]. To the best of our knowledge, only one method has been published for the quantification of varenicline in human plasma, using HPLC–MS/MS preceded by LLE [26], which has then been applied for the study of varenicline pharmacokinetics [27,28].

For the present work, high specificity was required to minimize the risk of interferences with compounds from the matrix (human plasma) and to discriminate between the chemically related analytes. High sensitivity was needed to ensure low limits of quantification (LLOQ), allowing to confirm abstinence from smoking, and wide calibration ranges, allowing to detect overdosage. Moreover, short analysis time and simultaneous quantification of the compounds are highly preferred. Therefore, the ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) allows to combine the advantages of the UPLC, such as increased resolution and sensitivity, decreased analysis time and solvent consumption [29] and the high detection specificity and sensitivity of the MS/MS. Thus, this methodology provides the most sensitive and specific method today available for the analysis of drugs and their metabolites.

The objective of the present work was to develop and validate a rapid and sensitive UPLC–MS/MS method for the simultaneous determination of smoking cessation drugs in human plasma and further apply it to samples of participants from a clinical study on smoking cessation.

2. Experimental

2.1. Chemicals and reagents

Varenicline, its internal standard (IS) (CP-533,633) and nicotine were kindly provided by their manufacturers: Pfizer (Groton, USA) and Siegfried (Zofingen, Switzerland), respectively. Cotinine, cotinine-d3 (1 mg/mL solution in methanol) and nicotine-d4 (100 μg/mL solution in methanol) were purchased from Sigma–Aldrich (Buchs, Switzerland), *trans*-3′-hydroxycotinine from Chemos (Regenstauf, Germany), and *trans*-3′-hydroxycotinine-d3 (1 mg/mL solution in methanol) from Toronto Research (Ontario, Canada). Biosolv® UPLC-grade acetonitrile, formic acid, methanol and isopropanol were bought from Chemie Brunchwig (Basel, Switzerland), Lichrosolv® 37% hydrochloric acid and 85% ortho-phosphoric acid from Merck (Darmstadt, Germany), 25% ammonium hydroxide solution, ammonium formate and ammonium acetate puriss p.a. for mass spectrometry from Sigma–Aldrich (Buchs, Switzerland). Ultrapure water was obtained from a Milli-Q® RG with a QPAQ2 column system (Millipore, MA, USA). All chemicals were of analytical grade. For the preparation of calibrators and quality control (QC) samples and the evaluation of matrix effects, more than 10 different batches of human plasma were obtained from the hospital's blood transfusion center (CHUV, Lausanne, Switzerland), that were tested for the presence of nicotine and/or metabolites prior to their use.

2.2. Equipment

The LC system consisted of a Waters Acquity UPLC instrument equipped with a binary pump and a 96-vial autosampler (Waters, Milford, MA, USA). Chromatographic separations were performed on a HILIC BEH column (2.1 × 100 mm, 1.7 μm) (Waters) equipped with a HILIC BEH cartridge (2.1 × 10 mm, 1.7 μm). Column temperature was set at 40 °C and the autosampler was kept at 8 °C. The chromatographic system was coupled to a triple quadrupole MS (TQD) (Waters) equipped with an ESI interface operated in positive ionization mode. Data acquisition handling and instrument control were performed by the Masslynx software version V4.1 (Waters).

2.3. Stock and working solutions

Standard stock solutions of all analytes each at 1 mg/mL in methanol were stored at −20 °C. Working solutions at 50 μg/mL were obtained by the dilution of the stock solutions with 0.01 N HCl. Calibrators and QC samples were prepared independently at different concentrations in analyte-free plasma, which was fortified with freshly prepared dilutions of the working solution in 0.01 N HCl at 0.05, 0.5 and 5 μg/mL. A single IS working solution at 1 μg/mL containing nicotine-d4, cotinine-d3, *trans*-3′-hydroxycotinine-d3 and CP-533,633 in 0.01 N HCl was prepared. All working solutions were stored at −20 °C until required for analysis.

2.4. Sample preparation

An aliquot of plasma sample (500 μL) was fortified with 50 μL of IS working solution. The mixture was then diluted with 500 μL of 4% H_3PO_4, followed by vortex mixing. The acidified plasma was then treated by SPE, using an Oasis MCX 10 mg 96-well extraction plate. The SPE plates were conditioned and equilibrated with 500 μL acetonitrile and water, respectively. 1000 μL of diluted sample was loaded onto the SPE plate and allowed to flow by gravity, after which a slow vacuum was applied. Plates were washed with

Table 2
Extraction recovery, matrix effect and process efficiency for nicotine, metabolites and varenicline extracted from human plasma.

Analyte	Concentration (ng/mL)		Extraction recovery (%, n = 6, CV%[a])		Matrix effect (%, n = 6, CV%[a])		Process efficiency (%, n = 6, CV%[a])	
	Low	High	Low	High	Low	High	Low	High
Nicotine	10	500	86 (8)	94 (4)	92 (6)	79 (6)	79 (3)	74 (4)
Nicotine-d4	100	100	91 (10)	91 (3)	92 (7)	84 (4)	83 (3)	76 (4)
Cotinine	10	500	87 (5)	70 (3)	103 (6)	106 (3)	89 (3)	84 (3)
Cotinine-d3	100	100	82 (5)	75 (3)	105 (4)	97 (3)	86 (3)	94 (3)
trans-3′-Hydroxycotinine	10	500	54 (10)	49 (13)	102 (5)	98 (6)	55 (12)	48 (15)
trans-3′-Hydroxycotinine-d3	100	100	52 (9)	51 (15)	108 (4)	105 (2)	56 (12)	53 (15)
Varenicline	10	250	92 (11)	83 (4)	136 (6)	120 (6)	125 (6)	99 (5)
CP-533,633	100	100	91 (7)	88 (4)	130 (8)	122 (5)	118 (3)	108 (4)
Nicotine/Nicotine-d4	10	500	96 (4)	104 (1)	102 (2)	94 (3)	97 (2)	98 (3)
Cotinine/Cotinine-d3	10	500	106 (3)	94 (3)	97 (4)	95 (3)	103 (3)	89 (4)
trans-3′-Hydroxycotinine/ *trans*-3′-hydroxycotinine-d3	10	500	104 (5)	107 (6)	95 (4)	93 (4)	98 (3)	99 (4)
Varenicline/CP-533,633	10	250	101 (5)	94 (4)	105 (3)	98 (2)	106 (6)	92 (2)

[a]CV%: Coefficient of variation.

1000 μL of 2% formic acid solution followed by 1000 μL acetonitile. The analytes were then eluted with 250 μL of acetonitrile/25% ammonium hydroxide (90:10, *v*/*v*) followed by 250 μL acetonitrile. After each step a slow vacuum was applied. The elution was vortex mixed and 5 μL were directly injected into the UPLC–MS/MS system.

2.5. UPLC–MS/MS conditions

The most suitable chromatographic conditions were achieved at a flow rate of 0.4 mL/min with a mobile phase composed of 10 mM ammonium formate buffer pH 3 (solution A) and acetonitrile with 0.1% formic acid (solution B), using the following gradient program: 95% of B maintained for 1.8 min, gradient to 70% of B from 1.8 to 2.0 min, hold at 70% of B from 2.0 to 3.4 min. The gradient was followed by recondition with 95% of B until 8.0 min, as requested for HILIC columns.

Detection was performed on a triple quadrupole MS detector, operated with an ESI interface in positive ionization mode. Quantification was achieved using multiple reaction monitoring (MRM) of the transitions *m*/*z* 163→130 (nicotine), 177→80 (cotinine), 193→80 (*trans*-3′-hydroxycotinine), 212→169 (varenicline) for the analytes and 167→134 (nicotine-d4), 180→80 (cotinine-d3), 196→80 (*trans*-3′-hydroxycotinine-d3) and 240→197 (CP-533,633) for the IS. These transitions were previously reported in other publications for the detection of the studied compounds [8,17,26]. Nitrogen was used as desolvation gas at a flow rate of 800 L/h and a temperature of 400 °C, and argon as collision gas at a flow rate of 0.2 mL/min. Source temperature was set at 150 °C and capillary voltage at 3 kV. Dwell time for each ion was 50 ms. Table 1 presents the optimized cone voltages and collision energies, together with the *m*/*z* ratios and the MRM transitions chosen for all the compounds.

2.6. Method validation

The method validation was based on the recommendations of the Société Française des Sciences et Techniques Pharmaceutiques (SFSTP) [30] and on the guidelines for Bioanalytical Method Validation published online by the Food and Drug Administration (FDA) [31].

2.6.1. Selectivity and carry-over

Method selectivity was tested by analyzing 10 blank plasma batches from different sources for interfering peaks. Possible carry-over effects were assessed by injecting solvent (acetonitrile) and plasma samples (n = 3) after the highest calibrator at 500 ng/mL for nicotine and varenicline and 1000 ng/mL for cotinine and *trans*-3′-hydroxycotinine.

2.6.2. Matrix effects, extraction recovery and process efficiency

Matrix effects were assessed quantitatively at low (10 ng/mL) and high concentration levels (500 ng/mL for nicotine and its metabolites, 250 ng/mL for varenicline) and at 100 ng/mL for the IS based on the approach of Matuszewski [32]. Three different series were processed: a neat standard solution of the analytes and IS in the SPE elution solvent (acetonitrile/25%ammonium hydroxide (90:10, *v*/*v*)) (A), duplicates of 6 analyte-free plasma extracted and fortified with the analytes and IS after extraction (B) and duplicates of 6 analyte-free plasma fortified with the analytes and IS before extraction (C). The matrix effect was evaluated for each analyte and IS by calculating the ratio of the mean peak area in the presence of the matrix (samples fortified after extraction) to the peak area in absence of the matrix (neat standard) and expressed in percentage (ME = B/A). The recovery of the extraction from the matrix was determined by comparing the mean peak areas of samples fortified before extraction (C) to samples fortified after extraction (B) (RE = C/B). The overall process efficiency, taking into account the ME and the RE, was assessed by calculating the ratio of the mean peak areas of samples fortified before extraction to the peak area in the neat standard (PE = C/A). For all three parameters, the variability between the different plasma batches was evaluated and expressed as coefficient of variation (CV%). A value inferior or equal to 15% was considered satisfactory. The same parameters and respective coefficients of variation were calculated considering the IS-normalized peak areas for each analyte.

2.6.3. Trueness and precision

Three validation series were performed on independent days to examine trueness and precision of the analytical procedure. Calibrators were initially prepared in duplicate at 8 levels (1, 2, 5, 25, 50, 100, 500, and 1000 ng/mL for nicotine and metabolites and 1, 2, 5, 25, 50, 100, 250, and 500 ng/mL for varenicline). QC samples were prepared in quadruplicate at 8 levels (same as calibrators) covering the expected range of concentrations, according to previously measured levels in human plasma [6,33–35]. Finally, 3 levels in duplicate for nicotine and 4 levels in duplicate for metabolites and varenicline were chosen to build the calibration curves, which were freshly prepared each day. Results were based on the peak area ratio between the drug and its respective IS. QC samples were analyzed against the calibration curve of the same series and the trueness of each concentration level was expressed as percentage of the theoretical value. Precision was estimated with variances of repeatability (within-run variance) and intermediate precision

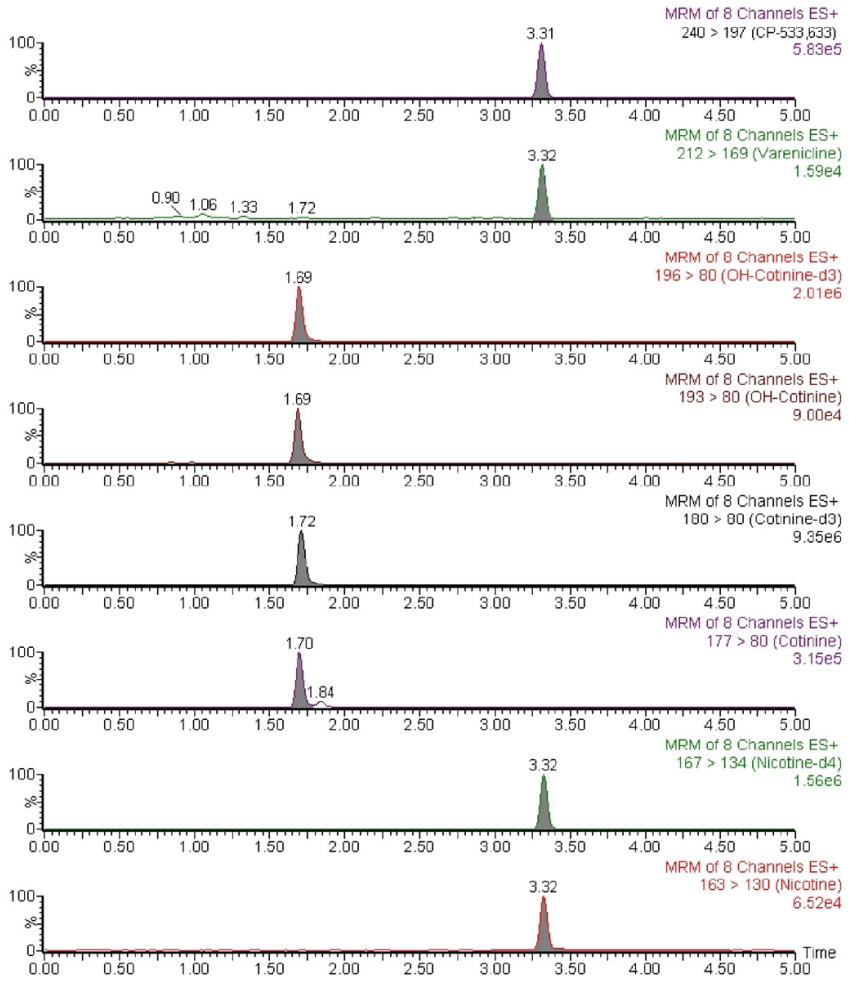

Fig. 1. Multiple reaction monitoring chromatogram of a QC plasma sample at 3 ng/mL (cotinine, varenicline), 6 ng/mL (*trans*-3′-hydroxycotinine, nicotine) and 100 ng/mL IS.

(sum of within-run and between-run variances), expressed as coefficient of variation (CV%) [30]. The LLOQ for each analyte was determined by the lowest QC concentration with a trueness and precision of ±20%, according to the above mentioned guidelines. Accuracy profiles within the acceptance limit ($\lambda = \pm 30\%$), and with confidence interval ($\alpha = 0.05$) were built for each analyte in the dosing range. The linearity of the method was equally assessed using a linear regression model which fitted the recalculated QC concentrations *vs.* theoretical concentrations [30].

2.6.4. Stability

The effect of different storage conditions on the stability of the compounds in plasma was assessed. Blank plasma from 5 different batches was fortified with the compounds of interest at low (5 ng/mL) and high (850 ng/mL for nicotine and metabolites, 450 ng/mL for varenicline) concentrations and quantified after storage at ambient temperature and at 4 °C up to 72 h, after one to three freeze–thaw cycles and after one, three and eight months at −20 °C. The postpreparative stability was assessed by leaving the processed samples for 24 h and 48 h at 8 °C before reanalysis. The variations in drug concentrations were expressed as percentage of the initial concentration found in the samples analyzed after preparation. The coefficients of variation (CV%) of the set of 5 plasma batches were also calculated for each compound and concentration level. A variation in plasma concentration inferior to 20% and CVs inferior to 15% were considered satisfactory.

3. Results and discussion

3.1. Solid-phase extraction

The sample preparation was based on a SPE procedure using a mixed-mode sorbent which combines hydrophobic interactions and cation-exchange, being thus suitable for the extraction of basic drugs from plasma. The use of different organic solvents as well as different washing steps was investigated. Satisfactory results regarding recovery, repeatability and selectivity were obtained by using ACN as organic solvent and 2% formic acid in water as washing solution. Recoveries were comprised between 70% and 94%, with the exception of *trans*-3′-hydroxycotinine and

Table 3
Assay validation parameters for nicotine, metabolites and varenicline.

Analyte	Concentration (ng/mL)	Trueness (%)	Precision	
			Repeatability (%)	Intermediate precision (%)
Nicotine	2	107.4	6.3	7.4
	5	106.5	4.6	11.3
	25	86.2	3.2	12.7
	50	96.3	4.6	11.8
	100	98.0	5.5	11.4
	500	112.0	5.8	13.5
	1000	113.6	7.7	15.3
Cotinine	1	97.8	8.8	8.8
	2	102.7	6.1	6.4
	5	104.4	6.6	9.6
	25	109.3	4.5	6.4
	50	107.3	3.7	8.2
	100	108.2	4.8	5.8
	500	97.7	3.9	6.5
	1000	90.6	5.0	5.0
trans-3′-Hydroxycotinine	2	103.6	9.9	10.9
	5	96.2	10.2	10.2
	25	96.3	5.9	8.5
	50	99.2	7.2	9.9
	100	99.9	8.5	8.5
	500	104.5	4.3	9.1
	1000	107.0	7.1	7.7
Varenicline	1	108.2	12.3	15.9
	2	99.2	10.9	10.9
	5	103.5	8.7	9.7
	25	102.3	1.9	4.6
	50	103.4	7.0	8.4
	100	101.7	9.0	9.0
	250	101.2	4.2	4.4
	500	96.1	4.6	4.6

trans-3′-hydroxycotinine-d3 for which recoveries were of 49–54%, probably due to their polar nature and thus to a poor retention on the SPE cartridge. However, the repeatability of results was satisfactory in all cases (CVs equal or below 15% for analytes and equal or below 6% for the analyte/IS ratio) (Table 2). No evaporation and reconstitution step was required and 5 μL of the SPE elution were directly injected into the UPLC–MS/MS system.

3.2. Optimization of chromatographic and MS/MS conditions

Preliminary separation assays have been performed using reversed phase chromatography (Acquity BEH C_{18} columns obtained from Waters) and a satisfactory separation was obtained. However, given the polar character of the compounds, the retention was poor and the percentage of acetonitrile at the beginning of the gradient was too low to allow for a sufficient desolvatation [36]. This resulted in a poor sensitivity and in estimated LLOQs that were not satisfactory for the purpose of the method. Given that varenicline is prescribed in small dosage (0.5 or 1 mg twice a day) [6], plasma levels were expected to be low: between 0.1 and 28.3 ng/mL in a population pharmacokinetic analysis [6]. Moreover, we aimed to use the procedure also for confirming abstinence from smoking in the study population. Therefore LLOQs for the studied compounds should ideally be low.

The HILIC technology involves the partitioning of an analyte between an organic mobile phase mainly polar and a water-enriched layer of the mobile phase, partially immobilized on the stationary phase [37]. The main advantages are an increased retention of polar compounds, enhanced sensitivity due to the high organic content in the mobile phase and possibility of directly

Table 4
Stability of nicotine, metabolites and varenicline in human plasma ($n = 5$) and after extraction.

Nominal conc. (ng/mL)	Nicotine		Cotinine		*trans*-3′-Hydroxycotinine		Varenicline	
	5	850	5	850	5	850	5	450
	Var%[a] (CV%[b])	Var%[a] (CV%[b])	Var%[a] (CV%[b])	Var%[a] (CV%[b])	Var%[a] (CV%[b])	Var%[a] (CV%[b])	Var%[a] (CV%[b])	Var%[a] (CV%[b])
Room temperature, 24 h	0 (5)	5 (6)	3 (9)	2 (5)	6 (4)	3 (9)	18 (7)	0 (5)
Room temperature, 72 h	7 (2)	1 (2)	0 (8)	−1 (4)	12 (7)	5 (4)	3 (2)	−3 (5)
4 °C, 72 h	0 (3)	3 (3)	−2 (6)	−3 (5)	9 (7)	7 (2)	−3 (7)	−4 (3)
Freeze/thaw, cycle 1	6 (4)	6 (1)	3 (9)	3 (5)	13 (5)	6 (3)	2 (4)	−3 (3)
Freeze/thaw, cycle 2	7 (5)	7 (3)	3 (7)	1 (5)	13 (14)	5 (3)	3 (6)	−4 (6)
Freeze/thaw, cycle 3	11 (3)	8 (4)	0 (5)	0 (4)	15 (6)	7 (2)	10 (8)	−4 (3)
Storage −20 °C, 1 month	5 (8)	7 (5)	−1 (3)	−1 (4)	7 (10)	2 (6)	−3 (3)	−6 (5)
Storage −20 °C, 3 months	−1 (8)	−5 (5)	3 (2)	0 (3)	2 (5)	3 (3)	9 (3)	−7 (4)
Storage −20 °C, 8 months	−3 (9)	−5 (2)	−3 (2)	−1 (2)	10 (8)	2 (1)	6 (6)	−3 (2)
Postpreparative 8 °C, 24 h	1 (4)	5 (2)	−2 (7)	5 (2)	−1 (6)	7 (3)	6 (9)	3 (6)
Postpreparative 8 °C, 48 h	−1 (3)	−1 (4)	3 (7)	4 (5)	4 (2)	4 (7)	12 (4)	0 (4)

[a] Var%: Variations in drug concentrations, expressed as percentage of the initial concentration found in the samples analyzed after preparation.
[b] CV%: Coefficient of variation.

Table 5
Plasma levels of nicotine, metabolites and varenicline measured in the study population.

	n	Calibrated range (ng/mL)	Quantified range (ng/mL) (6, 32–34)	Study samples (ng/mL) median (range)
Nicotine	406	2–500	1–100	17.5 (0–52)
Cotinine	406	1–1000	1–1000	251.5 (0–818)
trans-3′-Hydroxycotinine	406	2–1000	1–500	79 (0–314)
Varenicline	199	1–500	1–30	3 (0–20)

injecting the extraction organic elution onto the chromatographic column [25]. HILIC has been shown appropriate for hydrophilic bases at acid pH, using acetonitrile and ammonium formate buffers [25]. Therefore, to set up the chromatographic parameters a scouting gradient from 95% to 50% acetonitrile was used, testing buffers (ammonium formate, ammonium acetate) at different concentrations (5 mM, 10 mM) and pHs (from 2.8 to 5). The addition of different organic modifiers in the aqueous phase (3% of methanol or isopropanol) was also tested, in the attempt to obtain changes in retention and selectivity.

However, changes in pH or in buffer concentration did not significantly influence the selectivity of the separation, reflecting similar polarities and ionization between the compounds. Thus, cotinine and *trans*-3′-hydroxycotinine ($\log P = 0.34$ and -0.5; pKa = 4.5 and 4.5, respectively), coeluted, as well as nicotine and varenicline ($\log P = 1.09$ and 1.13; pKa = 8.2 and 8.8, respectively) [38,39]. The addition of 3% of methanol or isopropanol to the aqueous phase led to a complete separation of the compounds (data not shown). However, it was observed that organic modifiers interfered with the HILIC mechanism of retention, leading to a poor peak shape of nicotine. Thus, no organic modifier was used and instead, 0.1% of formic acid was added to acetonitrile to improve peak shapes, as previously described [25]. Overall, a significant increase in sensitivity was obtained with HILIC columns compared to reverse phase columns, which was in agreement with other published reports [40]. The most suitable chromatographic conditions were thus achieved with a mobile phase composed of 10 mM ammonium formate buffer pH 3 (solution A) and acetonitrile with 0.1% formic acid (solution B), using a gradient program from 95% of B until 70% of B and an overall time of 8.0 min. The average retention times of the analytes (t_R) are listed in Table 1 and a multiple reaction monitoring chromatogram of a QC plasma sample containing the compounds at 3 ng/mL (cotinine, varenicline), 6 ng/mL (*trans*-3′-hydroxycotinine, nicotine) and the IS at 100 ng/mL is shown in Fig. 1.

The high selectivity of an MS/MS system allows a successful quantification of the compounds, although their separation is not complete. This was further confirmed in our study by testing signal suppression in the two coeluting pairs, by keeping one substance at a fixed concentration (100 ng/mL) and increasing the other one up to 500 ng/mL (for nicotine and varenicline) and up to 1000 ng/mL (for cotinine and *trans*-3′-hydroxycotinine). No significant signal suppression was observed in the two pairs of coeluting compounds (CVs inferior to 5%) (data not shown). Moreover, no cross-talk was observed between the compounds when each analyte was injected separately and all MRM transitions were followed.

Deuterated internal standards, which allow to compensate for signal alterations due to matrix components and for variability in the extraction procedure [31,41], were used for nicotine and its metabolites. Varenicline is a newly developed drug and no deuterated standard was available during the development of the present procedure. Therefore, a structurally related compound (CP-533,633) which coeluted with varenicline was used, kindly supplied by the manufacturer together with varenicline.

Cone voltage and collision energy were optimized by directly infusing into the MS/MS detector a solution of each analyte at 1 μg/mL in 0.01 N HCl, at a flow of 10 μL/min and in combined mode with the mobile phase (30% of A and 70% of B). Cone voltage was tested in MS scan mode (values between 5 and 50 eV) and the value corresponding to the highest signal intensity was retained. The fragmentation spectrum was obtained in product scan mode and collision energies (between 5 and 50 eV) corresponding to the daughter ions with the highest abundance were retained.

3.3. Validation

3.3.1. Selectivity and carry-over

No peaks from endogenous compounds were observed at the drugs retention time in any of the blank plasma extracts evaluated. However, in some blank plasma of non-smokers we observed traces of nicotine and cotinine, maybe due to passive exposure to environmental tobacco smoke (ETS) or to nicotine intake through dietary sources, as previously reported [8]. Nevertheless, the peak areas of nicotine and cotinine in blank plasma were not significant, corresponding to less than 20% of the concentration at LLOQ. However, it was difficult to evaluate the presence of carry-over by injecting blank plasma after the highest calibrator. Therefore, acetonitrile was injected after the highest calibrator ($n = 10$) and no carry-over of the studied compounds was observed. Moreover, no significant cross-talk was observed between the IS and the parent compounds.

3.3.2. Matrix effects, extraction recovery and process efficiency

A limitation of the MS ESI mode of detection is the presence of matrix effects, meaning a signal enhancement or suppression by endogenous compounds present in the biological matrix and potentially coeluting with the analytes [42].

The quantitative assessment of interfering endogenous plasma components revealed slight matrix effects ranging from 79% for nicotine to 136% for varenicline. However, when the ratios analyte/IS were considered, matrix effects were comprised between 93% and 105%, showing that the use of individual coeluting IS corrected for these effects. Moreover, more important than the absolute matrix effects, is a reduced variability of these effects between the different plasma batches. With the present extraction procedure this aim was achieved, with variabilities between the 6 different plasma sources inferior to 8% for the substances alone and inferior to 4% for the ratio analyte/IS. Process efficiencies, which consider the combined effects of extraction recovery and of matrix effects, were also found to be satisfactory, ranging between 89% and 106% (for the analyte/IS ratio), with CVs inferior to 6%. All results are presented in Table 2.

3.3.3. Trueness and precision

Three validation series were performed on three different days and 8 calibrators in duplicate were initially used for each compound, covering the range from 1 to 1000 ng/mL for nicotine and its metabolites and from 1 to 500 ng/mL for varenicline. Determination of the most suitable calibration was performed by testing different regression models. Calibration curves of 3 and 4 levels in duplicate were selected, taking into account the LLOQ obtained for each compound: 2, 25, and 500 ng/mL for nicotine; 1, 25, 500, and 1000 ng/mL for cotinine; 2, 25, 500, and 1000 ng/mL for *trans*-3′-hydroxycotinine and 1, 25, 250, and 500 ng/mL for varenicline. Calibration curves were fitted for all compounds by a linear regression with a weighting factor of $1/x^2$.

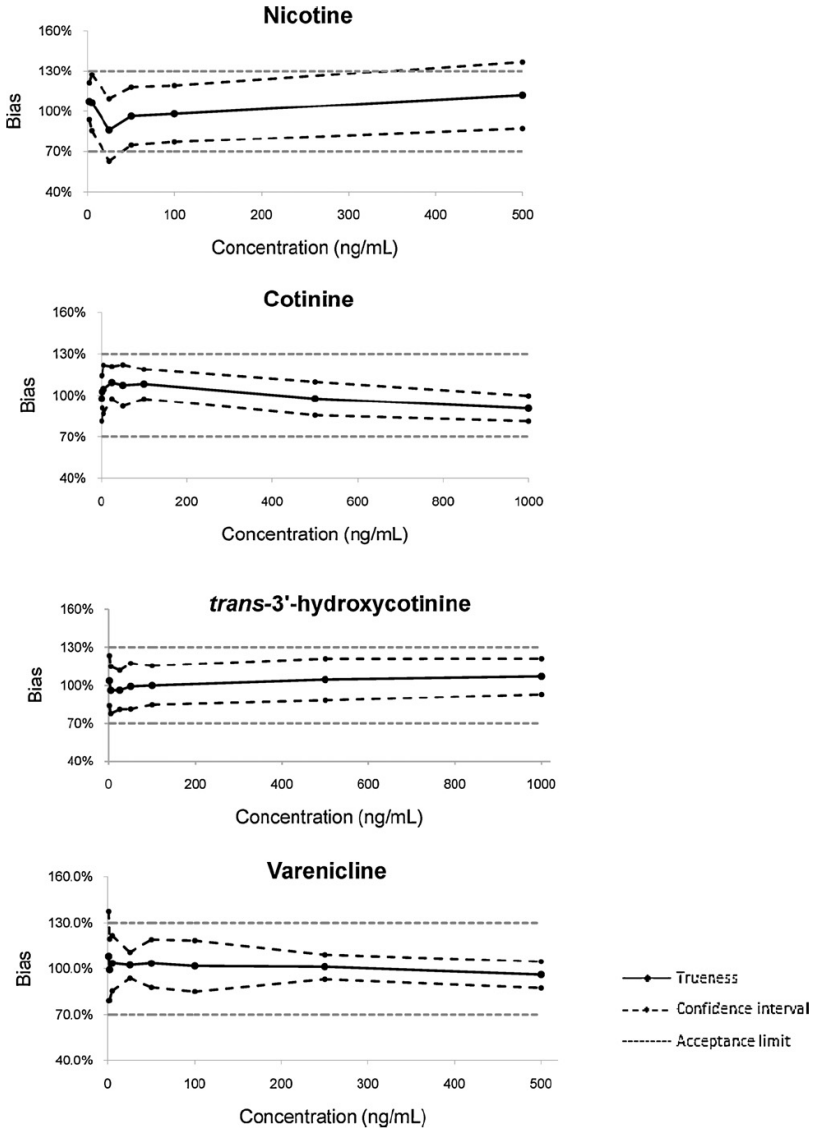

Fig. 2. Accuracy profiles for nicotine, metabolites and varenicline within the acceptance limit ($\lambda = \pm 30\%$), and with confidence interval ($\alpha = 0.05$).

The trueness, repeatability and intermediate precision of the back calculated QC samples are reported in Table 3. For all QC samples, the determined trueness met the acceptance criteria of ±15% (LLOQ ± 20%) with values between 86.2% and 113.6%. Moreover, repeatability and intermediate precision were within the required limits of ±15% (LLOQ ± 20%) with CVs within 1.9–12.3% and 4.4–15.9%, respectively. The accuracy profiles for each analyte in the dosing range are shown in Fig. 2. It has to be mentioned that the accuracy profile of nicotine at the concentration levels 25 and 500 ng/mL passed the ±30% limit, thus for this compound an acceptance limit of ±35% can be set, which is still suitable for the intended purpose of the analytical procedure [43].

To assess the linearity of the method, the following slopes 1.127, 1.069, 0.912 and 0.966, and intercepts −5.58, −3.694, 7.108 and 2.411 were obtained for nicotine, cotinine, *trans*-3′-hydroxycotinine and varenicline, respectively. The corresponding determination coefficients were 0.985, 0.993, 0.994 and 0.996, indicating that the procedure was linear for the tested compounds.

3.3.4. Stability

The stability of the analytes in plasma at room temperature and at 4 °C was ascertained up to 72 h. The variation over time of each analyte was within a range of ±15% of the initial concentration, with the exception of varenicline after 24 h at room temperature, which exceeded 15% but remained below 20% (18% of gain at low concentration). This indicates that the drugs are stable in plasma at room temperature and at 4 °C for at least three days. Furthermore, stability in plasma was equally demonstrated after three

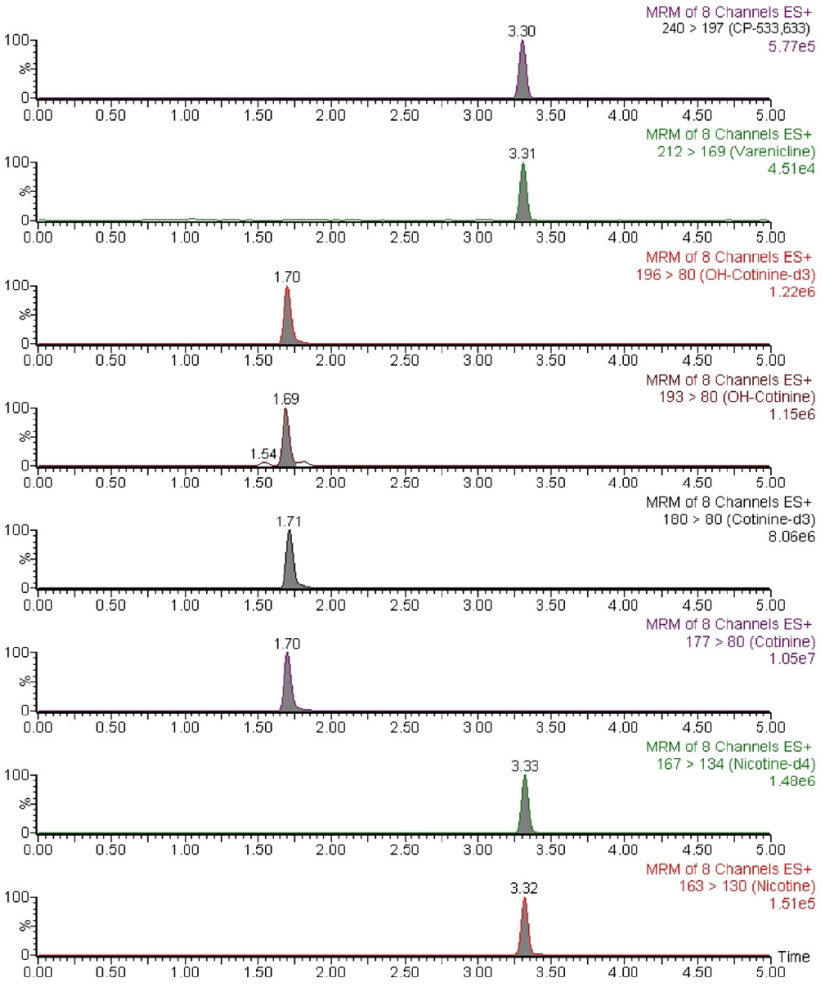

Fig. 3. Chromatogram of a participant's plasma at the beginning of the study, while smoking and having started varenicline treatment (nicotine 15 ng/mL, cotinine 185 ng/mL, *trans*-3′-hydroxycotinine 130 ng/mL and varenicline 9 ng/mL).

freeze and thaw cycles and long term storage stability at −20 °C was confirmed after one, three and eight months, with variations in drug levels comprised within ±15% of the initial concentrations. Additionally, the postpreparative stability of the compounds in the extracted samples, assessed by keeping the vials for 24 h and 48 h at 8 °C before reanalysis, yielded satisfactory results (below 12% of variation from the nominal value). Moreover, the CVs of all stability tests were below 14%. All results of the stability tests are presented in Table 4.

4. Clinical application

The analytical procedure was applied to determine plasma levels of the studied compounds in 194 subjects participating in a clinical study on smoking cessation [44]. The program consisted of a 3-month follow-up composed of smoking cessation counselling and pharmacological treatment prescription (nicotine replacement therapy or varenicline) and a 6-month conclusion visit. Blood sampling was performed at baseline when participants were still smoking, and served for the quantification of nicotine and its metabolites. One month after smoking cessation, blood sampling was used to quantify varenicline in subjects prescribed with this drug and to confirm abstinence in the same subjects. A last blood sample at the 6-month follow-up visit allowed confirmation of abstinence in all subjects that completed the study. Overall, 406 plasma samples were analyzed and results are presented in Table 5. All measured plasma concentrations were within the calibrated ranges and in accordance with plasma levels of these drugs previously reported in literature [6,33–35]. A representative chromatogram of a participant in the study, while smoking and who had already started varenicline treatment, is shown in Fig. 3 (nicotine 15 ng/mL, cotinine 185 ng/mL, *trans*-3′-hydroxycotinine 130 ng/mL and varenicline 9 ng/mL). These results are used to study the pharmacogenetics of varenicline and nicotine and to confirm abstinence

from smoking at the above mentioned time points in the clinical study.

5. Conclusion

A sensitive UPLC–MS/MS method using HILIC for the simultaneous quantification of nicotine, its metabolites cotinine and *trans*-3′-hydroxycotinine and varenicline was developed and validated according to the SFSTP and FDA guidelines. The drugs were extracted from plasma using a simple SPE procedure, which removed efficiently endogenous compounds from the matrix. The analytical procedure allows a fast quantification (in 3.4 min) of the four compounds over a usually measured concentration range, which was confirmed by applying the method to real samples of a smoking cessation study.

Conflict of interest

The authors declare no conflict of interest.

Role of the funding source

The funding sources have no role on the design, conduct, and reporting of the study or in the decision to submit the manuscript for publication.

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