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Implementation of a cost-effective HPLC/UV-approach for medical routine quantification of donepezil in human serum

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ABSTRACT

A novel, simple, specific and sensitive high performance liquid chromatography (HPLC) assay for the detection and quantification of donepezil in serum of demented patients has been developed and validated. The analytical procedure involves an offline serum preextraction using solid phase extraction (SPE) cartridges (Oasis® HLB, Waters Co). The chromatographic analyses were performed on a Dionex HPLC system with a Phenomenex Luna Phenyl-Hexyl analytical column, and a mobile phase with the two components 0.02 mol/l phosphate buffer and acetonitrile. The flow rate was 0.4 ml/min. For the detection of donepezil three different UV wavelengths were used as an interference-control check. Interference tests between donepezil and 100 of the most commonly used concomitant medications allow quantification of donepezil under the polypharmaceutical conditions of the daily clinical routine. The retention time for donepezil was 12.1 min. The method was validated according to the guidelines of the Society of Toxicology and Forensic Chemistry (GTFCh): The calibration curve was linear over a concentration range from 5 to 160 ng/ml ($n = 8/r^2 > 0.999$). No endogenous compounds were found to interfere with the analyte, which was shown by retention times for the comedication most often prescribed to demented patients. The method had an accuracy of >85%. Intra- and inter-assay coefficients of variation were <6% and <8%, respectively, at three different concentrations. The limit of quantification (LOQ) and the limit of detection (LOD) were found to be 6.1 and 1.7 ng/ml for donepezil. Application of the method to patient serum samples discovered that concentrations suggested as "therapeutic" in the literature may only be reached either by high, off-label dosages or by utilization of inhibitory metabolic effects of the comedication.

1. Introduction

The concentration rather than the administered dose defines the efficacy of a drug. Hence its determination is important to rationally support physicians' decisions on drug dosage adjustments. For the last few years we set up our clinical pharmacological laboratory by developing analytical methods for the quantification of psychotropic drugs in serum of patients [1,2]. Now, we would like to report the development of such methods for the quantification of antidementive drugs beginning with donepezil.

More than 24 million people worldwide suffer from dementia. If current demographic trends continue, the number will rise substantially in the coming years, since geriatric people have a higher risk for the disease [3]. So far two groups of drugs are approved for the treatment of dementia (see Fig. 1). Donepezil, galantamine and rivastigmine are cholinesterase inhibitors, that are licensed for the treatment of patients with mild to moderate Alzheimer's disease. Memantine is a noncompetive glutamate (NMDA) receptor antagonist and is licensed for moderate to severe Alzheimer's disease. All these drugs just delay the progression of the disease, the dementia cannot be cured. At present the aim of the treatment is to maintain functioning and quality of life as long as possible [4]. It is therefore of particular interest to benefit from the full pharmacological potential of donepezil.

Classical TDM (therapeutic drug monitoring) uses the determination of drug serum concentrations to avoid adverse drug effects (ADE). In a further step TDM supports the choice of dosage of

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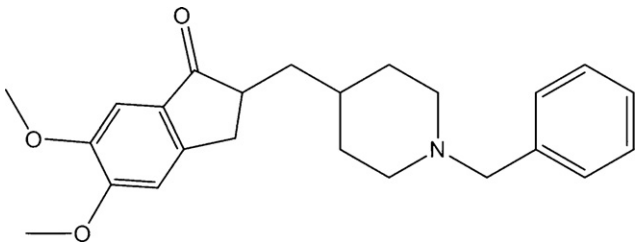

Fig. 1. Chemical structure of donepezil.

a drug to improve effectiveness. Moreover, our laboratory does not just return a determined drug concentration as a plain value but rather together with a clinical pharmacological comment that relates the concentration to both a therapeutic and a dose-related reference range [5,6]. The therapeutic reference range gives a concentration above which the desired drug effect can be expected and another, higher concentration above which the risk of undesired drug effects overcomes the potential benefit of the drug. The dose-related reference range gives the concentration that can be expected in a "normal patient", i.e. the patient population of the phase II clinical trial that established the total clearance of the drug [6]. Especially geriatric, multimorbid, and demented people with polypharmacy are suspected for significant deviations of their serum drug concentrations from "normal" patients. These differences are caused by an age-dependent decrease in functioning of the excretion organs, by drug–drug interactions in polypharmacy, by genetic abnormalities in drug metabolism and by the individual way of life. This information is returned to the treating physician in the clinical pharmacological comment thereby supporting the physician's choice of necessary comedications for treatment of the individual patient.

Various analytical methods were described for the quantitative determination of donepezil in biological samples. They include high performance liquid chromatography (HPLC) with ultraviolet (UV) and fluorescence (FL) detection as well as liquid chromatography (LC) with mass spectrometry (MS) and tandem mass spectrometry (LC–MS/MS) [7–11]. Earlier publications often described labor- and time-consuming sample preparation techniques using liquid–liquid extraction to clean up the antidementive drugs from their serum matrix before chromatographic separation of the sample [8]. Nowadays more and more LC methods are developed with expensive mass spectrometric detection techniques [11]. However, most of the institutions in laboratory medicine that analyze drug concentrations for medical routine analyses still use HPLC/UV systems as a basis for their determinations.

It was the aim of the present investigation to develop and validate a simple, fast and cost-effective HPLC/UV method suitable for routine analysis of donepezil.

2. Experimental

2.1. Reagents and chemicals

Donepezil hydrochloride was kindly supplied by Eisai GmbH (Frankfurt, Germany). Ammonium chloride, ammonium formate, and ammonium hydroxide were obtained from Sigma–Aldrich (Seelze, Germany). Acetic acid, acetone, formic acid, methanol, ortho-phosphoric acid, potassium dihydrogen phosphate and sodium acetate were all HPLC or reagent grade from Merck KGaA (Darmstadt, Germany); Acetonitrile was Ultra Gradient HPLC grade from J.T. Baker (Deventer, Holland). Drug-free human serum was prepared from whole blood drawn from healthy volunteers (Bezirksklinikum Regensburg, Germany). Water used for preparation of standards, as well as for buffers and the mobile phases, was prepared using a Milli-Q Gradient A10 water purification system from Millipore (Bedford, MA, USA). Two commercially available control sera with a low and a high concentration were obtained from Recipe GmbH (Munich/Germany). External quality control sera came from INSTAND e.V. (Düsseldorf, Germany) six times a year.

2.2. Instrumentation

SPE cartridge eluates were evaporated using a vacuum infrared dancer (Hettich AG, Baech, Switzerland) associated with a Penguin Cold Trap and a PC2004 vario vacuum pump. Centrifugation was performed using a Megafuge 2.0 R from Heraeus Sepatech GmbH (Osterode, Germany).

The analytical procedure was performed on a HPLC system from Dionex GmbH (Idstein, Germany) consisting of a GINA 50T auto sampler, an isocratic low pressure gradient pump M580G, a programmable column heater TCC 100 and a variable wavelength photodiode array detector (DAD) 320S. All analyses were performed with a Phenomenex Security Guard Cartridge Phenyl 4×2.0 mm precolumn coupled to the analytical column. Analytical columns tested were purchased directly from the manufacturer: Thermo Betasil C-6 250×4.6 mm (5 μm) from Thermo Scientific (Karlsruhe, Germany), MZ LiChrospher 100 RP-18 150×4.6 mm (5 μm) from MZ Analysentechnik (Mainz, Germany), Phenomenex Luna Phenyl-Hexyl 150×3 mm (3 μm) from Phenomenex LTD (Aschaffenburg, Germany), Waters Sunfire C18 150×3 mm (3.5 μm) from Waters LTD (Eschborn, Germany), and Agilent Zorbax SB-Phenyl 150×4.6 mm (3.5 μm) from Agilent Technologies (Böblingen, Germany).

The chromatograms were monitored and integrated by the Chromeleon® software Version 6.8 SP2 Build 2284.

2.3. Preparation of stock and calibration solutions

Donepezil hydrochloride stock solutions (100,000 ng free base/ml) were prepared in ethanol–water 40:60 (V/V) and stored frozen at −20 °C for 6 months without measurable decompositions. Working solutions (100, 1000 and 10,000 ng/ml) for the calibration standards were diluted daily with water.

Drug-free serum from healthy volunteers was spiked daily by mixing working solution directly on top of a solid phase extraction cartridge to achieve "low", "middle" and "high" concentrations with final concentrations of donepezil 0 (blank), 20, 40 and 80 ng/ml. The concentrations chosen covered the therapeutic reference range of the antidementive drug.

Liophylisates of Recipe control samples "low" and "high" as well as lyophilisates of INSTAND interlaboratory tests were reconstituted with water according to the production specification. Afterwards they were handled the same way as the calibration and patient samples.

2.4. Sample collection and processing for analysis under medical routine

7.5 ml venous blood was collected from patients taking antidementive drugs using 21-gauge butterfly needles (Multifly Set) connected to serum monovettes, both from Sarstedt (Nuembrecht, Germany). The venipuncture was carried out after pharmacokinetic steady state had been reached, about 15 days for donepezil ($\sim 5 \times t_{1/2}$; $t_{1/2} = 70$ h [21]), usually in the morning 1 h before next pill intake [6]. Following centrifugation ($1800 \times g$, 15 min, 25 °C) serum was transferred to cryogenic tubes and stored frozen at −20 °C until analysis.

2.4.1. Sample preparation by solid phase extraction (SPE)

Liquid–liquid extraction according to the procedure described previously [8] was compared to the solid-phase extraction. Oasis HLB cartridges 1 cc/30 mg (Waters Corporation, Eschborn, Germany) were activated with 1 ml of methanol followed by 1 ml of water. One cartridge for each specimen was then loaded with either 1 ml of serum calibration solution, 1 ml of serum patient sample or 1 ml of quality control sample (daily both Recipe "low" and "high" concentration, INSTAND interlaboratory test samples once every third month). All SPE-steps were realized with a Waters vacuum station coupled to a vacuum pump set to 20 mbar.

After loading, each of the Oasis HLB cartridges designated for quantification of donepezil was washed with 1 ml of 5% methanol in aq. dest. (V/V, washing step 1) followed by 1 ml of 0.025 mol/l ammonium chloride buffer (pH 9)/methanol, 50:50 (V/V, washing step 2). The waste fluids were discarded.

A buffer solution was used to elute the analyte from the cartridges: 1 ml of 0.025 M ammonium formate buffer pH 2.5/methanol 50:50 (V/V) for donepezil. The eluates were evaporated to dryness for 20 min at 37.5 °C and reconstituted with 100 μl of HPLC mobile phase. Following a brief vortex and centrifugation at 500 × *g* for 3 min, the supernatants were transferred to brown small-volume glass vials with 0.1 ml conical inserts from Varian GmbH (Darmstadt, Germany) for automated injection into the HPLC system.

2.5. Analytical chromatography

Mobile phases were based on 0.02 mol/l potassium dihydrogen phosphate buffer and acetonitrile, the pH value was adjusted with ortho-phosphoric acid. The pump delivered a constant flow of 0.4 ml/min, the injection volume was 35 μl, column temperature was set to 30 °C, and the DAD recorded at 210 nm. Peak purity was double checked by detection at two further wavelengths, 235 and 268 nm (see Fig. 2). Calibration standards and patient samples were analyzed in duplicates.

To determine the standard calibration curves, drug serum concentrations were plotted against peak heights. Chromeleon® calculated a linear regression analysis for each calibration curve with no weighting.

2.6. Validation

All methods were validated according to the guidelines of GTFCh (Society of Toxicology and Forensic Chemistry) in consideration of ISO 5725 (International Organization of Standardization) [12], FDA (US Food and Drug Administration) guidances [13] and ICH (International Conference on Harmonization) requirements [14].

2.6.1. Calibration curve

Calibration curves were documented over a concentration range of 5, 10, 20, 40, 80, 160 ng/ml ($n = 8$) for donepezil. A linear regression analysis was calculated that yielded the linear regression equations and the coefficient of certainty r^2.

2.6.2. Limit of quantification, limit of detection

For each component the limit of quantification (LOQ) and the limit of detection (LOD) were calculated based on signal-to-noise ratio of 10:1 and 3:1, respectively, by analysing 10 different concentrations evenly distributed over a concentration range from 1 ng/ml to 160 ng/ml ($n = 4$). The values were verified by linear regression analysis over the data points. The determination of LOD and LOQ was carried out according to DIN 32645 in consideration of $p \leq 0.01$ (significance level) and $k = 3$ (analytical uncertainty ≤ 33%).

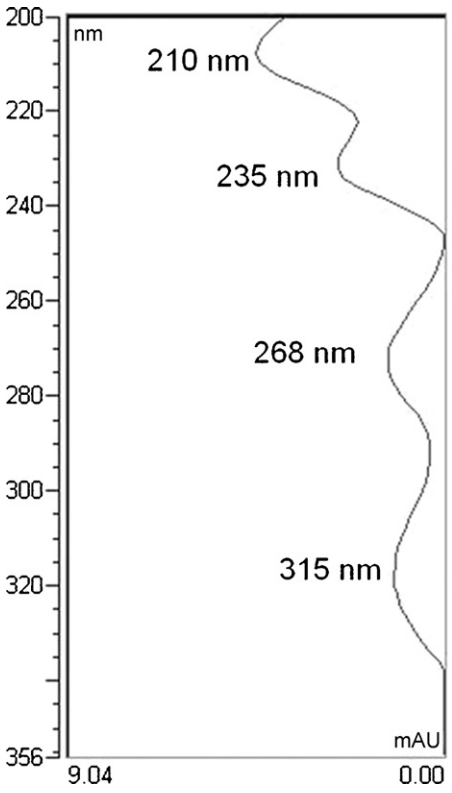

Fig. 2. The UV absorptions maxima of donepezil.

2.6.3. Recovery/extraction efficiency

The absolute recovery of the antidementia drugs in serum was determined for two different concentrations. Spiked drug serum samples were compared to aqueous drug solutions, set to 100% ($n = 6$).

In addition, the extraction efficiency was determined in the way to exclude any matrix effects. Therefore blank serum was spiked with the analyte before and after SPE. The percent extraction efficiency from serum was expressed as the mean analyte peak height of serum samples spiked with donepezil before SPE divided by the mean analyte peak height of serum samples with donepezil added after SPE ($n = 6$).

2.6.4. Precision

Precision was assessed by repeated analyses ($n = 4$) on the same day and on six different days of serum samples containing 5, 40, 160 ng/ml of donepezil. Intraassay variation is expressed as coefficient of variation (CV) of the four measurements on the day with the highest variation, interassay variation as CV of all 24 measurements.

2.6.5. Accuracy

Accuracy of the method was assessed by repeated analyses of Recipe quality control samples and the INSTAND interlaboratory test samples ($n = 2$/series of analyses). Data are given as coefficient of variation of the deviations from the expected target value.

2.6.6. Selectivity

Interference tests were performed with 100 drugs identified as the medications most often prescribed to dementia patients. The prescription data base of the AGATE (working group for pharmacotherapy in psychiatric hospitals) pharmacovigilance program [15] was evaluated for this purpose. For each method the agents were just solubilized in mobile phase without SPE before analyzed by HPLC/UV. Interference was defined as a retention time within

Table 1
Data of precision.

Antidementia drugs	Conc. (ng/ml)	Intra-assay (CV in %)	Inter-assay (CV in %)
Donepezil	5	3.0	7.4
	40	2.1	6.4
	160	1.1	6.0

a range of ±1 min of the donepezil retention time. Agents with retention times within that range were then solubilized in drug-free serum and pre-treated with SPE before analyzed by HPLC/UV again (Flurazepam and Oxcarbazepine – see Table 3).

2.7. Modifications for routine analysis

After regular use of all methods in medical routine analysis a routine-suitable strategy was developed to identify peak impurities.

2.8. Statistical analysis

Data are presented as mean ± SD and coefficient of variation (CV). Differences between two conditions were statistically validated by Student's *t*-test. $p < 0.05$ was regarded as statistically significant.

2.9. Therapeutic drug monitoring (TDM)

The method was applied to routine TDM of blood specimens obtained from patients suffering from dementia according to our procedure: After quantification the drug concentration is returned together with a clinical pharmacological comment relating the concentration to two reference ranges thereby providing the treating physician with information on the pharmacological state of treatment and individual pharmacological peculiarities of the patient [5]. The therapeutic reference range for donepezil (30–75 ng/ml) was taken from the consensus TDM guideline that compiles the best validated therapeutic reference ranges for psychoactive drugs available in the international literature [16]. Dose-related reference ranges were calculated according to the equation given in Fig. 3 [6]. The total clearance of of donepezil was taken from Tiseo et al. [17]. Clinical pharmacological commenting was done using the internet platform KONBEST ([18]; www.konbest.de).

3. Results

3.1. Stability of serum specimens

There was no degradation of donepezil within six month of storage in the dark at −20 °C.

3.2. Sample preparation development

The solid phase technique was characterized by advantages over liquid/liquid extraction such as simplicity (less handling procedures, less risk of handling errors, no need for separation of lipophilic from hydrophilic phases which is highly difficult in microscale procedures), expenditure of time (approximately 10 min for 20 specimens in solid-phase extraction versus 30 minutes for 20 specimens in liquid/liquid extraction), lower consumption of solvents (40 ml methanol versus 80 ml *n*-heptane for 20 specimens), and recovery (donepezil 20 ng/ml: 53.2 ± 2.0% versus 28.7 ± 10.8% ($n = 6$); donepezil 80 ng/ml: 56.0 ± 0.9% versus 35.1 ± 6.9% ($n = 6$)). Taking into account the environmental and economic aspects, the solid-phase extraction was chosen.

3.2.1. Removing impurities

Tuning the pH-value and tuning the ratio of organic solvent to water in the mobile phase were applied to remove impurities on the Oasis HLB sorbent. Two washing steps were developed to remove salts and proteins (washing step 1) and to eliminate acids, neutral compounds and weak hydrophobic bases (washing step 2) from the sorbent (Fig. 4). There was no elution of donepezil between pH 9.0 and 7.0 in a 0.025 mol/l ammonium formate buffer/methanol, 50:50 (V/V), it started between pH 7.0 and 5.0 and reached its maximum at pH 2.5 (Fig. 5). pH lower than 2.5 resulted in even higher elution, however also for other substances thereby decreasing selectivity. Therefore, the final washing procedure consisted of 1 ml of 5% methanol in aq. dest. pH 7.0 (V/V, washing step 1) and 1 ml of 0.025 mol/l ammonium chloride buffer (pH 9)/methanol, 50:50 (V/V, washing step 2). Donepezil was eluted from the cartridge with 1 ml of 0.025 mol/l ammonium chloride buffer (pH 2.5)/methanol, 50:50 (V/V).

3.3. Analytical chromatography

Various chromatographic columns were tested during the prevalidation experiments. The best peak shape, resolution and reasonable retention times were achieved with the Phenomenex Luna Phenyl-Hexyl analytical column. Varying the pH and the amount of acetonitrile of the analytical buffer solution yielded in the chromatogram a peak for each of the four antidementia drugs with a reasonable retention time (<17 min). However, an isocratic HPLC method proved to be advantageous for robust, exact and reproducible analysis of antidementia drugs close to the limit of quantification due to a sensitive chromatographic baseline at UV detection wavelengths <230 nm. For donepezil a KH_2PO_4 buffer–acetonitrile composition of 75:25 (V/V) and pH 2.7 were chosen that led to the retention time 12.1 ± 0.2 min (C.V. = 1.6%). Experiments with more lipophylic mobile phases showed faster analytical runs and decreased retention times, but then the serum noise in the beginning of a chromatogram caused overlapping peaks and corrupted exact quantifications. Typical chromatograms of blank serum and serum spiked with donepezil are presented in Fig. 6A1 as well as an example from the medical routine in Fig. 6A2.

The UV absorption spectrum of donepezil showed maximal extinction at 210 nm, and three smaller extinction maxima at wavelengths of 235 nm, 268 nm, and 315 nm (Fig. 2). 210 nm was used as detection wavelength, two further wavelengths at 235 and 268 nm were used to double check for peak impurities.

3.4. Validation

The method was validated with the UV detection wavelength of 210 nm. Validation with the two wavelengths used to double check for peak impurities (235 nm and 268 nm) yielded similar results.

3.4.1. Calibration curve

The calibration curve was linear between 5 and 160 ng donepezil per ml with a linear regression equation of $y = (0.088 \pm 0.002)x - (0.164 \pm 0.131)$, where y represents the peak-height and x the concentration of the analyte. The coefficient of certainty r^2 was >0.999.

3.4.2. Limit of quantification, limit of detection

The limit of quantification (LOQ) and the limit of detection (LOD) were determined for donepezil to be 6.1 and 1.7 ng/ml, respectively.

3.4.3. Recovery

Recovery of donepezil was 55.7 ± 1.5% at 160 ng/ml and 58.6 ± 2.0% at 5 ng/ml. Extraction efficiency was 77.3 ± 3.1% at 160 ng/ml and 88.8 ± 2.6% at 5 ng/ml.

Calculation the dose-related reference range for donepezil [6]:

$$c = \frac{D_e \cdot f \cdot F}{\tau \cdot (Cl_F \pm SD)}$$

c (medial steady state serum concentration of donepezil) in ng/ml, D_e (daily dosage of donepezil) = x mg,
Cl/F (Total clearance ± standard deviation/bioavailability) = 14738 ml/h ± 1762 [17], τ (dose frequency) = 24 h [21],
f (correction factor: donepezil salt (tablet) into free base)= 0,91 [21].

1.) Upper limit of the dose-related reference range:

$$c_1 = \frac{D_e \cdot f}{\tau \cdot (Cl_F + SD)/F} = \frac{D_e \cdot 1000000 \ \frac{ng}{mg} \cdot 0.91}{24\,h \cdot (14738 \ \frac{ml}{h} - 1762)} = D_e \cdot 2.92 \ \frac{ng}{mg \cdot ml}$$

2.) Lower limit of the dose-related reference range:

$$c_2 = \frac{D_e \cdot f}{\tau \cdot (Cl_F - SD)/F} = \frac{D_e \cdot 1000000 \ \frac{ng}{mg} \cdot 0.91}{24\,h \cdot (14738 \ \frac{ml}{h} + 1762)} = D_e \cdot 2.30 \ \frac{ng}{mg \cdot ml}$$

Example: A patient is taking donepezil 5 mg/d since 4 weeks (pharmacokinetic balance).

- Upper limit of the dose-related reference range for 5 mg donepezil: 15 ng/ml
- Lower limit of the dose-related reference range for 5 mg donepezil: 11 ng/ml

Fig. 3. Calculation the dose-related reference range for donepezil.

3.4.4. Precision

The intra- and the inter-assay coefficients of variation were <3 and <8% of the mean, respectively, at three different concentrations (Table 1).

3.4.5. Accuracy

All results were in accordance with required target values of the Recipe quality control samples and the INSTAND interlaboratory test samples (within ±15%). Each method had an accuracy of >85% (Table 2).

3.4.6. Selectivity

An analysis of the AGATE data base for prescription manners in dementia patients yielded 100 medications most often prescribed to dementia patients (Table 3). Interference tests with these chemicals discovered just oxcarbazepine and flurazepam with

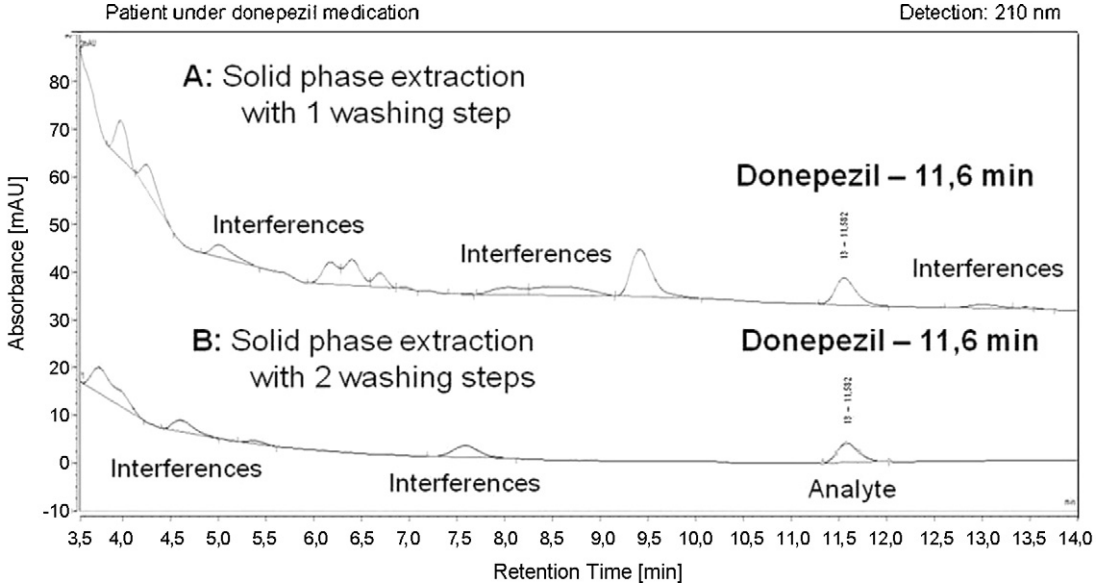

Fig. 4. Chromatograms of a patients' serum extract after solid phase extraction with (A) one washing step and with (B) two washing steps to remove interferences for exact quantifications of donepezil.

Table 2
Data of accuracy.

Donepezil–calibrator-concentration (ng/ml):	20	40	80
Measurand: Absolute peak height (mAU) at 210 nm			
Target value (S.D.), $n = 2$	1.396 (0.017)	3.160 (0.053)	6.860 (0.117)
Fresh, selfprepared quality control samples:	Low	Middle	High
Deviation from the target value (SD), $n = 2$	0.089 (0.012)	0.074 (0.021)	0.083 (0.025)
Bias %:	6.38	2.34	1.21
Recipe quality control samples:	Low	Middle	High
Deviation from the target value (SD), $n = 2$	−0.173 (0.018)	−0.238 (0.021)	−0.649 (0.093)
Bias %:	−14.40	−12.37	−12.04
Instand interlaboratory test samples:	Low	Middle	High
Deviation from the target value (SD), $n = 2$	−0.173 (0.018)	−0.238 (0.021)	−0.649 (0.093)
Bias %:	−14.40	−12.37	−12.04

retention times close to donepezil (Table 3). These two drugs, however, were cleaned from the patients' specimens during SPE sample preparation. No compounds were found to interfere with donepezil after SPE sample preparation.

3.5. Checking peak purity by detection at three wavelengths

Just in case a surprising interference would occur in the chromatogram in spite of the precautions described above, the HPLC system applied in our experiments will nevertheless allow a correct quantification of the analyte: Fig. 7 shows the determination of donepezil in a patientïs serum at three different UV wavelengths (at 210, 235 and 268 nm). The chromatogram was recorded at a time when the development of the SPE extraction technique was still in progress. Fig. 7 demonstrates at 210 nm two peaks which do partly overlap. The signal of an unknown drug from the patient's comedication with a high extinction coefficient at 210 nm overlaps the donepezil signal. Nevertheless, the peak could be evaluated because of simultaneous detection at two further wavelengths 235 and 268 nm by the variable wavelength photodiode array detector (DAD), which identified and confirmed a serum concentration of less than one third the concentration detected at 210 nm. This concentration was accepted as result of the analysis.

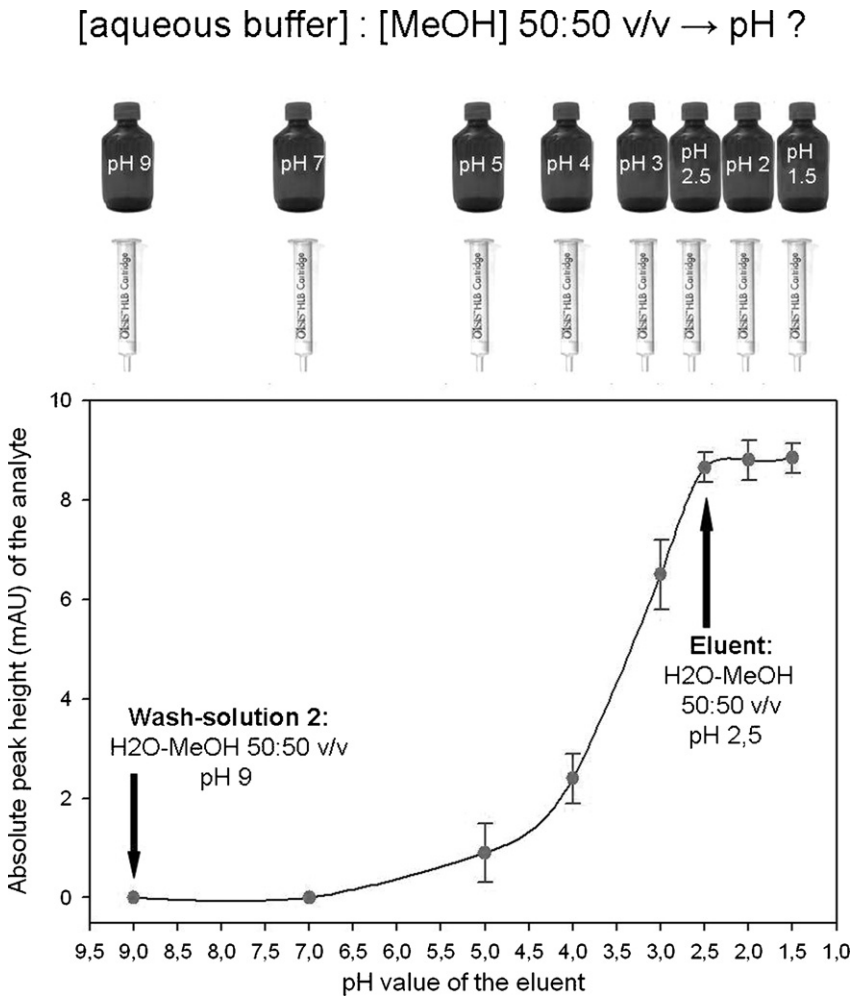

Fig. 5. Development of a SPE-method to obtain higher donepezil recovery (serum samples with donepezil 100 ng/ml, $n = 6$, UV detection wavelength 210 nm).

Table 3
Interference tests with over 100 of the most described medications for patients with dementia (database: ~5130 patients of the AGATE pharmacovigilance program) demonstrate the feasibility for routine analysis. (n.d. = not detected, d.p. = drug product).

Nr	Drug dissolved in mobile phase:	Number of patients:	Percent (%) of patients:	Retention times (min) without SPE! Detection at 210, 235 and 268 nm
	Donepezil	361	1.34	10.9 10.9 10.9
	Galantamine	261	0.97	2.7 2.7 2.7
	Rivastigmine	130	0.48	3.7 n.d. n.d.
	NAP 226-90			n.d.
	Memantine	253	0.94	n.d.
1	Acetylic salicylic acid	1229	23.96	7.2 7.2 7.2
2	Risperidone	1221	23.81	4.9 4.9 4.9
	9-Hydroxyrisperidone			5.2 5.2 5.2
3	Melperone	1215	23.69	6.9 6.9 6.9
4	Lorazepam	894	17.43	32.1 32.1 32.1
5	Pipamperone	820	15.99	2.9 2.9 2.9
6	Haloperidol	670	13.06	19.3 19.3 19.3
7	Furosemide	648	12.63	25.7 25.7 25.7
8	Omeprazole	584	11.39	8.5 8.5 8.5
9	Lactulose	539	10.51	n.d.
10	Mirtazapine	531	10.35	3.2 3.2 3.2
11	Carbamazepine	465	9.07	19.1 19.1 19.1
	Monohydroxycarbamazepine			n.d.
12	Citalopram	402	7.84	15.6 15.6 n.d.
	DM-Citalopram			13.7 13.7 n.d.
13	Ramipril	395	7.70	17.1 n.d. n.d.
14	Metoprolol	376	7.33	3.8 3.8 3.8
15	Valproic acid	372	7.25	n.d.
16	Olanzapine	361	7.04	6.9 6.9 6.9
	DM-Olanzapine			n.d.
	Ethylolanzapine			2.58 2.58 2.58
17	Enalapril	343	6.69	3.1 3.1 n.d.
18	Quetiapine	335	6.53	6.8 6.8 6.8
19	Levothyroxine	328	6.40	n.d.
20	Potassic salt	320	6.24	n.d.
21	Levodopa	247	4.82	n.d.
22	Bisoprolol	267	5.21	5.4 5.4 5.4
23	Insulin d.p.	218	4.25	n.d.
24	Allopurinol d.p.	243	4.74	21.7 21.7 21.7
25	Captopril d.p.	236	4.60	n.d.
26	Digitoxin d.p.	218	4.25	n.d.
27	Hydrochlorothiazide	181	3.53	4.6 4.6 4.6
28	Escitalopram	176	3.43	15.6 15.6 n.d.
29	Zopiclone	174	3.39	4.1 4.1 4.1
30	Clopidogrel d.p.	169	3.29	14.3 14.3 14.3
31	Biperiden	164	3.20	18.8 n.d. n.d.
32	Chlorprotixene	160	3.12	n.d.
33	Glimepiride d.p.	154	3.0	n.d.
34	Diazepam	141	2.75	7.9 7.9 7.9
	DM-Diazepam			21.6 21.6 21.6
35	Zolpidem d.p.	141	2.75	3.6 3.6 3.6
36	Sertraline	139	2.71	n.d.
	DM-Sertraline			42.8 42.8 n.d.
37	Theophylline	126	2.46	2.9 2.9 2.9
38	Folic acid d.p.	121	2.36	n.d.
39	Metamizole d.p.	119	2.32	n.d.
40	Diclofenac	116	2.26	4.3 4.3 n.d.
41	Metformin	111	2.16	2.0
42	Verapamil d.p.	109	2.13	n.d.
43	Ranitidine d.p.	92	1.79	2.3 2.3 2.3
44	Venlafaxine	90	1.75	5.8 5.8 5.8
	DM-Venlafaxine			3.4 3.4 3.4
45	Lamotrigine	83	1.62	3.7 3.7 3.7
46	Doxepin	71	1.38	15.7 15.7 15.7
	Nordoxepin			n.d.
47	Clozapine	70	1.36	4.7 4.7 4.7
	Norclozapine			3.7 3.7 3.7
	DM-Clozapine			3.5 3.5 3.5
48	Nifedipine d.p.	65	1.27	4.1 4.1 4.1
49	Simvastatin d.p.	58	1.13	6.9 6.9 6.9
50	Gabapentin	57	1.11	n.d.
51	Amisulpride	56	0.21	3.2 3.2 3.2
52	Oxazepam	56	0.21	27.0 27.0 27.0 n.d.
53	Desipramine	55	0.20	22.6 22.6 22.6 n.d.
54	Magnesium ion	55	0.20	n.d.
55	Sodium chloride	52	0.19	n.d.

Table 3 (*continued*).

Nr	Drug dissolved in mobile phase:	Number of patients:	Percent (%) of patients:	Retention times (min) without SPE! Detection at 210, 235 and 268 nm
56	Paroxetine	49	0.18	25.2 25.2 n.d. n.d.
57	Flunitrazepam	45	0.17	n.d.
58	Reboxetine	44	0.16	13.4 13.4 13.4
59	Amitriptyline	43	0.16	32.3 32.3 n.d.
60	Paracetamol d.p.	39	0.14	2.8 2.8 2.8
61	Haloperidol	37	0.14	19.3 19.3 19.3
62	Zuclopenthixole	36	0.13	22.6 22.6 22.6
63	Oxacarbazepine	35	0.13	11.5 11.5 11.5
	10-Hydroxycarbazepine			6.2 6.2 n.d. n.d.
64	Perazine	35	0.13	8.5 8.5 8.5
65	Propranolol	33	0.12	8.6 8.6 8.6
66	Levetiracetam	29	0.11	3.0 3.0 n.d.
67	Clonazepam	28	0.10	38.9 38.9 38.9
68	Trimipramine	27	0.10	35.9 35.9 35.9
69	Pantoprazole	27	0.10	6.9 6.9 6.9
70	Pirenzepine	27	0.10	2.6 2.6 2.6
71	Flupentixol	26	0.10	38.1 38.1 38.1
72	Jodid	25	0.09	n.d.
73	Primidone	22	0.08	4.6 4.6 n.d.
74	Fluoxetine	20	0.07	41.5 41.5 n.d.
	Norfluoxetine			n.d.
75	Aripiprazole	13	0.05	3.0 n.d. n.d.
	Dehydroaripiprazole			36.2 36.2 n.d.
76	Bromazepam	12	0.04	4.2 4.2 4.2
77	Fluphenazine	10	0.04	n.d.
78	Duloxetine	10	0.04	30.8 30.8 n.d.
79	Ziprasidone	9	0.03	12.6 12.6 12.6
80	Paliperidone	8	0.03	5.2 5.2 5.2
81	Clomipramine	6	0.02	n.d.
	Norclomipramine			n.d.
82	Clobazam	6	0.02	n.d.
83	Nortriptyline	6	0.02	26.8 26.8 n.d.
84	Amitriptylinoxide	5	0.02	40.5 40.5 n.d.
85	Phenytoine	5	0.02	22.3 22.3 n.d.
86	Flurazepam	4	0.01	11.6 11.6 11.6
87	Imipramine	4	0.01	25.8 25.8 25.8
88	Amlodipine	3	0.01	27.8 27.8 27.8
89	Mianserin	3	0.01	13.2 13.2 13.2
90	Fluvoxamine	1	0.00	21.7 21.7 21.7
91	Nitrazepam	1	0.00	24.4 24.4 24.4
92	Alprazolam	1	0.00	5.4 5.4 5.4
93	Sumatriptane	1	0.00	2.9 2.9 2.9
94	Dihydrocodeine	1	0.00	5.1 5.1 5.1
95	Nateglinide	1	0.00	n.d.
96	Bromperidol	1	0.00	n.d.
97	Chlordiazepoxide	1	0.00	n.d
98	Ethosuximide	1	0.00	4.3 n.d. n.d.
99	Pregabalin	1	0.00	n.d.
100	Bupropion	1	0.00	5.7 5.7 5.7
	Hydroxybupropion			4.2 n.d. n.d.
	Erythrobupropion			6.0 n.d. n.d.
	Threohydrobupropion			5.4 n.d. n.d.

Nr:	Drug in serum:	Number of patients:	Percent (%) of all patients:	Retention times (min) after SPE! Detection at 210, 235 and 268 nm
	Donepezil	361	1.34	12.1 12.1 12.1
63	Oxacarbazepine	35	0.13	n.d.
86	Flurazepam	4	0.01	n.d.

3.6. Therapeutic drug monitoring (TDM)

The method has been successfully applied to the routine therapeutic drug monitoring of antidementia drugs in patients. Fig. 8 shows 34 donepezil concentrations determined from patient serum samples plotted against the prescribed dose. Only 17 of these concentrations were found within the therapeutic reference range for donepezil, one was above and the remaining 16 were found below the therapeutic reference range. All of the 17 donepezil concentrations within and the one above the therapeutic reference range were classified too high in relation to the dose prescribed (above the dose related reference range, Figs. 8 and 9). The latter classification was explained in the clinical pharmacological comment by enzyme inhibition exerted by known enzyme inhibitors prescribed in the patients' comedications.

4. Discussion

The described isocratic HPLC/UV method enables a simple, cost effective, accurate and precise quantification of donepezil in serum. With just a few modifications this assay can be used for the TDM

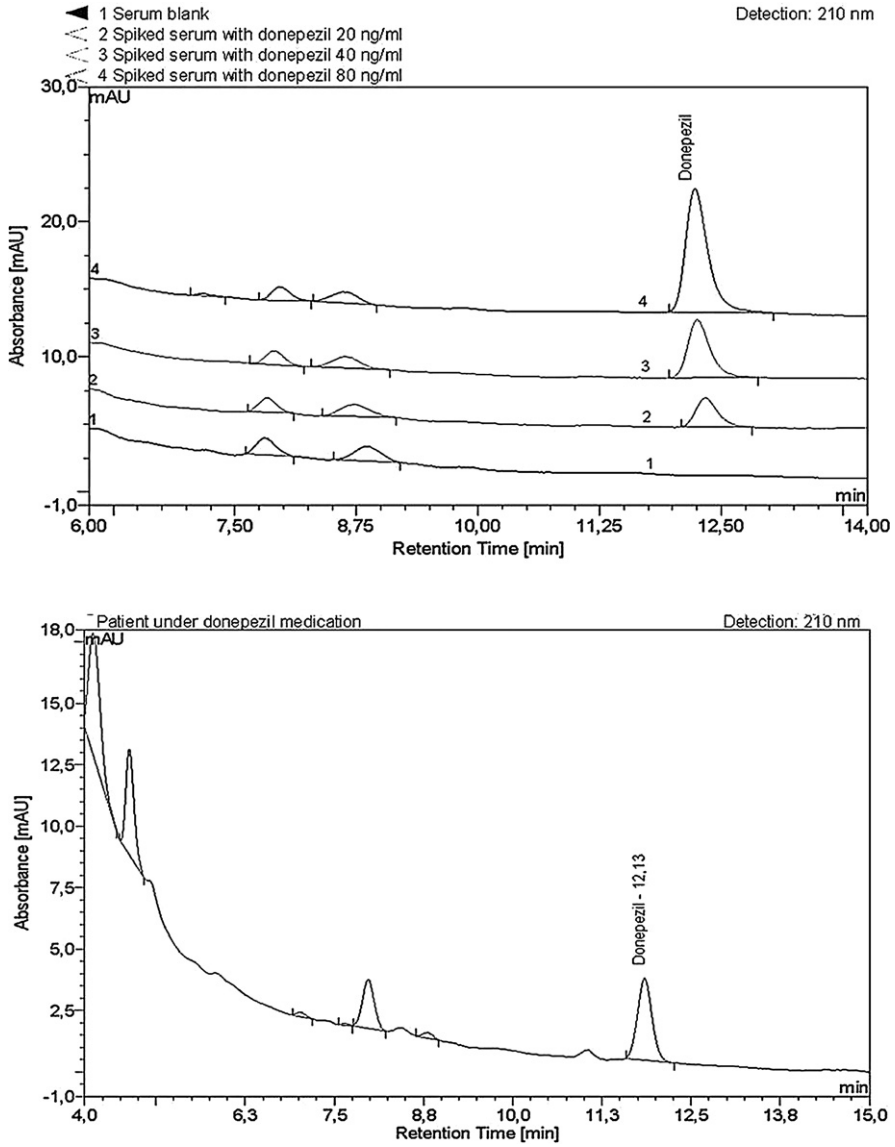

Fig. 6. Representative chromatograms of blank serum and serum spiked with (A1) donepezil (20, 40 and 80 ng/ml). Chromatogram (A2) obtained from a patient after a daily antidementia drug treatment. A1: donepezil calibration standards; A2: patient with a daily donepezil dose of 5 mg and a corresponding conc. of 27 ng/ml in serum.

of all antidementia drugs on one identical, analytical column and with the same mobile phase components. This will be described elsewhere.

4.1. Precolumn sample preparation development

Online column switching HPLC methods for spectrophotometric quantification of multiple drugs in a single analytical run are very suitable for medical routine analysis [1,2]. Serum samples are directly injected without precolumn extraction. Therefore these methods are indeed advantageous for economic reasons because they allow fast automated HPLC runs without labor- and time-consuming sample preparations. Unfortunately they are sensitive to interferences of signals originated by drug metabolites, the comedication and physiological biochemicals.

Oasis HLB cartridges from Waters were therefore chosen for a precolumn solid phase extraction sample clean-up. This technique is characterized by its simplicity, fastness, better recovery, and low consumption of solvents in comparison to liquid–liquid extractions. Furthermore SPE provides cleaner samples for HPLC analysis, which is quite often a problem when serum samples are directly injected in column-switching technique. Oasis HLB extraction cartridges are based on a hydrophylic–lipophylic-balanced, water-wettable, reversed phase sorbent. It composes of two monomers, a hydrophilic N-vinylpyrrolidone and a lipophilic divinylbenzene which provide a special reversed-phase capacity with enhanced retentions for polar analytes.

4.1.1. Removing of impurities

Variation the concentration of organic solvents and the pH values are two important variable factors by which interferences can be avoided and higher specificity for the analyte can be reached. The procedures described in Section 2.4.1 were the best compromise to simplify the complexity of the serum samples by removing interfering compounds (e.g. proteins, salts, drugs of the concomitant medications) that might poison the chromatographic column or affect the sensitivity of the antidementia drug detection, and to get acceptable extraction recoveries.

4.1.2. Strategies to avoid overlapping

Overlapping can never be completely avoided in HPLC runs of samples obtained from patients treated with a large number of

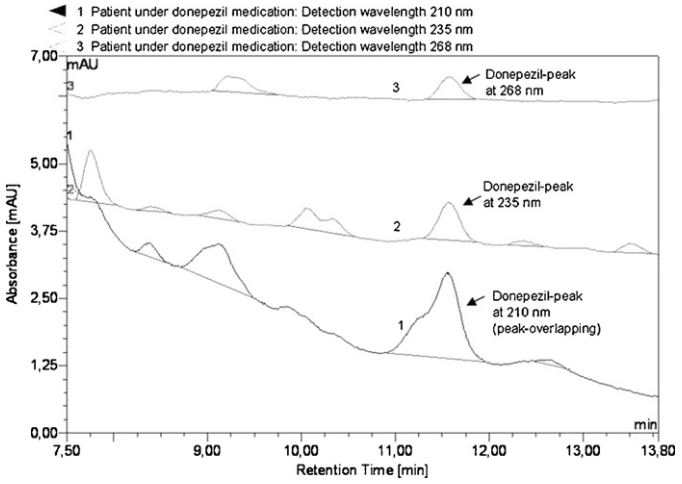

Fig. 7. Chromatogram with an overlapping donepezil peak at 210 nm: The peak purity at longer wavelengths (235 and 268 nm) confirmed a lower analyte concentration by around one third and therewith the advantage of UV detections at minimum three different wavelengths for exact quantifications.

medications (polypharmacy). It may occur at higher as well as at lower wavelengths. In some cases complete peak overlapping may occur that may not be visible in a chromatogram at all.

Different strategies were pursued in this research to avoid overlapping peaks. Firstly, donepezil was separated by solid phase extraction technique using Wasters Oasis HLB cartridges. It was possible to isolate the analyte from serum matrix components including drugs of the patientsĭ concomitant medications. In this context several washing fluids and eluants in different compositions and pH-values were tested and optimized. Furthermore, the detections of standard and analyte at three different UV wavelengths (see Section 2.5) proofed to be a practicable tool to verify peak purities which is a prerequisite for exact quantifications. In the daily routine the authors clear and release a drug concentration only if the value is similarly detected at least with two different detection wavelengths.

Today more and more laboratories use liquid chromatography methods with mass spectrometry detection. This technique is advantageous in terms of specificity and speed. It is, however, very cost-intensive (acquisition costs/highly qualified stuff) and in comparison with UV detection still more error-prone in quantifications. In addition HPLC/MS is susceptible against ion suppression and misinterpretations of samples containing several drugs with identical masses [19].

Table 4
Options for an upgrade of the donepezil method.

Antidepressant drugs	**Antiepileptic drugs**
Amitriptyline	Carbamazepine
Amitriptyline oxide	Lamotrigine
Bupropion	Phenytoin
Metabolite 1	Primidone
Metabolite 2	Sultiame
Metabolite 3	
Desipramine	**Antipsychotic drugs**
Citalopram	
Escitalopram	
Desmethylcitalopram	Clozapine
Doxepin	Norclozapine
Duloxetin	Flupentixol
Fluoxetin	Haloperidol
Fluvoxamine	Levomepromazine
Imipramine	Melperone
Maprotiline	Olanzapine
Mianserin	Methylolanzapine
Mirtazapine	Perazine
Nortriptyline	Quetiapine
Paroxetine	Risperidone
Reboxetine	9-Hydroxyrisperidone
Trimipramine	Zuclopentixol
Venlafaxine	Ziprasidone
Desmethylvenlafaxine	

4.2. Extension of these methods to the quantification of other drugs

Geriatric people are usually prescribed a larger number of drugs. This is particularly true for demented people, because they present a number of symptoms besides reduced cognition called dementia associated psychotic symptoms. The method presented in this paper may be used for quantification of these drugs as mentioned in Table 4 to offer a complete TDM for these patients.

4.3. TDM of antidementia drugs

For gerontopsychiatric routine the pharmacological interpretation of the individual patient drug concentrations is an important tool to alert for individual abnormalities such as drug/drug- or drug/food-interactions, gene polymorphisms (slow/rapid-metabolizer), intoxications, altered functions of the excretion organs (liver/kidney) by age and/or disease, compliance problems, etc.

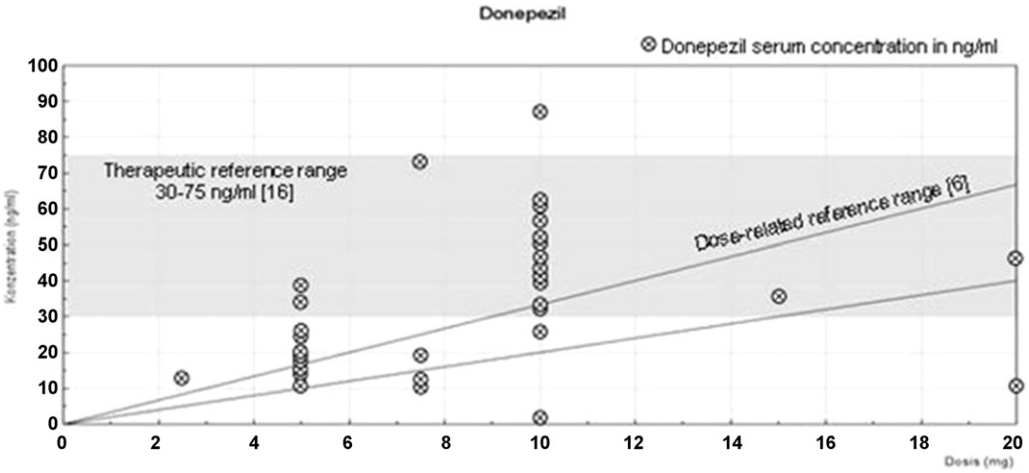

Fig. 8. The donepezil serum concentration of 34 patients is plotted against the daily dosage. Donepezil concentrations in accordance with the therapeutic reference range and the dose-related reference range are located at the intersection of both ranges.

Dose-related reference range [6]	Therapeutic reference range of donepezil: 30-75 ng/ml [16]			
	too low	match	too high	
too low	4 (12,1%)	-	-	4 (12,1%)
match	5 (15,2%)	4 (12,1%)	-	9 (27,3%)
too high	6 (18,2%)	13 (39,4%)	1 (3,0%)	20 (60,6%)
	15 (45,5%)	17 (51,5%)	1 (3,0%)	

Fig. 9. The nine fields table shows that just 4 of 33 patients had donepezil serum concentrations conform to the dose-related and the therapeutic reference range.

How valid are published therapeutic reference ranges of antidementia drugs? The AGNP (consortium for neuropsychopharmacology and pharmacopsychiatry) published a therapeutic reference range of 30–75 ng/ml for donepezil [16]. There is, however, only one study yet which demonstrates that patientsĭ improvement was better when the serum concentration of donepezil was above 50–75 ng/ml [20].

Using the dose-related reference range [6] our attention was drawn to the fact that donepezil serum concentrations measured under daily medical routine (Fig. 8) were influenced almost exclusively by the patients' polypharmacy: Inhibitory and inductive effects had a major impact on the metabolism of donepezil via the cytochrome-P_{450}–isoenzymes 2D6 and 3A4. It is of particular importance to see that not a single patient had reached the target concentration of 50–75 ng/ml suggested by Rogers et al. [20], least of all the therapeutic reference range listed by the AGNP [16] without inhibitory metabolic effects exerted by the patient's comedication.

5. Conclusion

The described procedure was the first assay to implement therapeutic drug monitoring of donepezil by HPLC/UV in medical routine analysis. The method reported here is simple, specific and sensitive. For economical reasons this assay has the capacity to be expanded to the qualitative and quantitative analysis of further drugs and their active metabolites relevant in the polypharmacy of demented patients, in particular other psychotropic drugs.

Case histories of the AGATE pharmacovigilance program demonstrate that most of the dementia patients with donepezil treatment are underdosed. In "normal" cases off-label doses up to 40 mg/d donepezil will be necessary to reach the therapeutic concentrations suggested by Rogers et al. [20]. Such an approach can be supported by TDM as suggested in this paper.

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References

[1] C. Greiner, C. Hiemke, W. Bader, E. Haen, J. Chromatogr. B 848 (2007) 391.
[2] C. Greiner, E. Haen, J. Chromatogr. B 854 (2007) 338.
[3] C.P. Ferri, M. Prince, C. Brayne, Lancet 366 (2005) 2112.
[4] C.H. Gleiter, H.-P. Volz, Antidementiva – Physiologie, Pharmakologie und klinische Anwendung, 1st ed., Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, 2008, ISBN: 3804723942.
[5] E. Haen, Psychopharmakotherapie 12 (2005) 138.
[6] E. Haen, C. Greiner, W. Bader, M. Wittmann, Nervenarzt 79 (2008) 558.
[7] S.L. Rogers, L.T. Friedhoff, Br. J. Clin. Pharmacol. 46 (1998) 1.
[8] N. Yasui-Furukori, R. Furuya, T. Takahata, T. Tateishi, J. Chromatogr. B 768 (2002) 261.
[9] H. Pappa, R. Farru, P.O. Vilanova, M. Palacios, M.T. Pizzorno, J. Pharm. Biomed. Anal. 27 (2002) 177.
[10] K. Nakashima, K. Itoh, M. Kono, M.N. Nakashima, M. Wada, J. Pharm. Biomed. Anal. 41 (2006) 201.
[11] Y. Lu, H. Wen, W. Li, Y. Chi, Z. Zhang, J. Chromatogr. Sci. 42 (2004) 234.
[12] L.D. Paul, F. Musshoff, Toxichem. Krimtech. 76 (2009) 142.
[13] Guidance for Industry on Biomedical Method Validation, 2001. http://www.fda.gov/cder/guidance/index.htm. (Stand: 1/8/10).
[14] ICH expert working group: Harmonised Tripartite Guideline – Validation of Analytical Procedures: Methodology, 1996, ICHQ2B, CPMP/ICH 281/95.
[15] E. Haen, Neurotransmitter 15 (2004) 34.
[16] P. Baumann, C. Hiemke, S. Ulrich, G. Eckermann, I. Gaertner, M. Gerlach, H.J. Kuss, G. Laux, B. Müller-Oerlinghausen, M.L. Rao, P. Riederer, G. Zernig, Pharmacophsychiatry 37 (2004) 243.
[17] P.J. Tiseo, R. Vargas, C.A. Perdomo, L.T. Friedhoff, Br. J. Clin. Pharmacol. 46 (1998) 55.
[18] A. Koestlbacher, E. Haen, Pharmacopsychiatry 41 (2008) 212.
[19] F.L. Sauvage, J.M. Gaulier, G. Lachatre, P. Marquet, Clin. Chem. 54 (2008) 1519.
[20] S.L. Rogers, L.T. Friedhoff, J.T. Apter, R.W. Richter, J.T. Hartford, T.M. Walshe, B. Baumel, R.D. Linden, F.C. Kinney, R.S. Doody, R.L. Borison, G.L. Ahem, Dementia 7 (1996) 293.
[21] SPC Aricept. Eisai Ltd., Hatfield, Herts, United Kingdom, 2009.