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Simultaneous determination of antidementia drugs in human plasma: Procedure transfer from HPLC–MS to UPLC–MS/MS

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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 ([$^2H_7$]-donepezil, [^{13}C,$^2H_3$]-galantamine, [$^{13}C_2$,$^2H_6$]-memantine, [$^2H_6$]-rivastigmine) were extracted from 250 μL human plasma by protein precipitation with acetonitrile. Chromatographic separation was achieved on a reverse phase column (BEH C18 2.1 mm × 50 mm; 1.7 μm) 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 Fig. 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.

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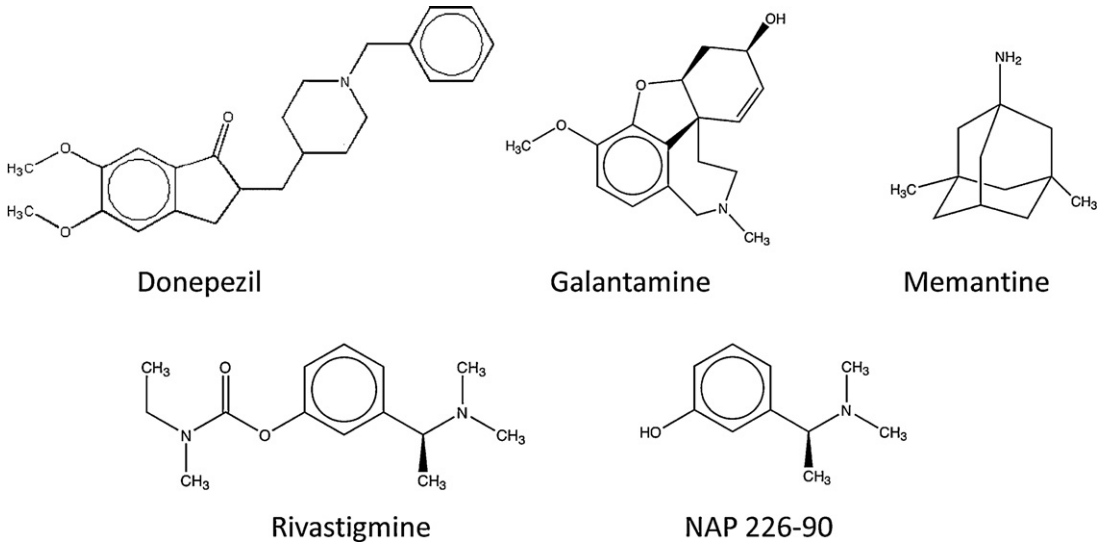

Fig. 1. Chemical structures of the analytes.

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 mm × 50 mm; 1.7 μm) (Waters) equipped with a BEH C18 cartridge (2.1 mm × 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 × *g* (12,610 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 16,000 × *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

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

	Parent (*m*/*z*)	Fragment (*m*/*z*)	Cone voltage (eV)	Collision energy (eV)	Dwell time (ms)	t_R[a] (min)
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] Retention time.

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 ($\lambda = \pm 30\%$) and with β-expectation tolerance intervals ($\beta = 90\%$) 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 (2.1 mm × 100 mm; 3.5 μm), a BEH C18 column (2.1 mm × 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

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)
[$^2H_7$]-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,$^2H_3$]-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)
[$^2H_6$]-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)
[$^{13}C_2$,$^2H_6$]-memantine	50	50	105 (9)	100 (4)	47 (6)	54 (10)	49 (12)	54 (12)
Donepezil/[$^2H_7$]-donepezil	3	250	98 (4)	105 (3)	99 (3)	103 (1)	96 (3)	109 (3)
Galantamine/[^{13}C,$^2H_3$]-galantamine	3	250	102 (3)	95 (2)	96 (3)	102 (3)	98 (2)	106 (2)
Rivastigmine/[$^2H_6$]-rivastigmine	0.6	40	99 (2)	101 (2)	99 (3)	98 (2)	98 (3)	98 (2)
NAP 226-90/[$^2H_6$]-rivastigmine	0.6	40	96 (2)	96 (1)	96 (3)	95 (1)	93 (3)	93 (2)
Memantine/[$^{13}C_2$,$^2H_6$]-memantine	3	250	105 (4)	104 (3)	97 (5)	98 (3)	102 (6)	103 (4)

[a] Coefficient of variation.

and a chromatographic profile of a QC plasma sample at the lower limit of quantification (LLOQ) is shown in Fig. 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, [$^2H_6$]-rivastigmine was used as IS after verification of the absence of signal suppression of rivastigmine on [$^2H_6$]-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 [$^2H_7$]-donepezil, and signal enhancement for memantine and [$^{13}C_2$,$^2H_6$]-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 [$^2H_7$]-donepezil, and 110% and 105% for memantine and [$^{13}C_2$,$^2H_6$]-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

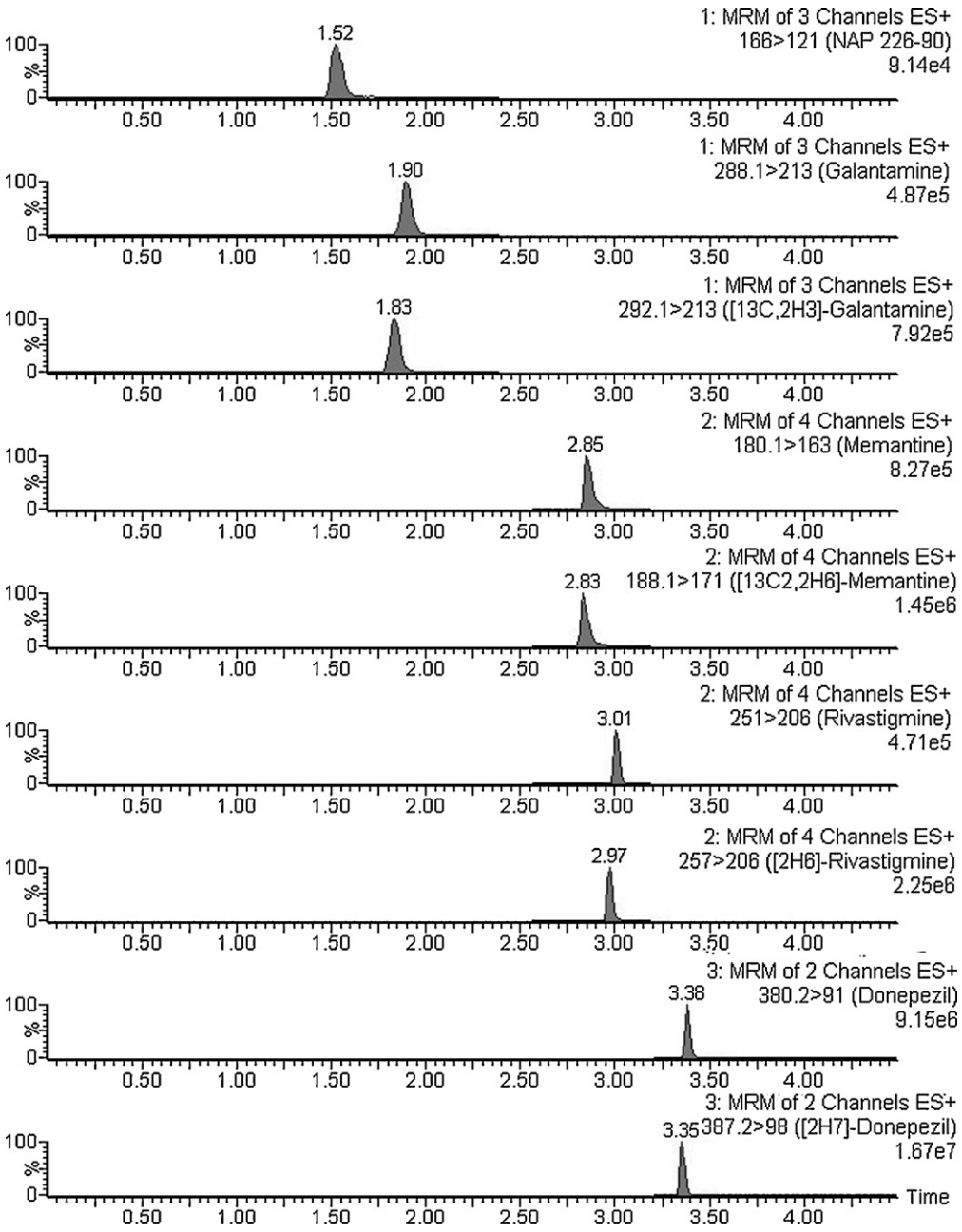

Fig. 2. Chromatogram of a QC plasma sample at LLOQ (1 ng/mL donepezil, galantamine, memantine, 0.2 ng/mL rivastigmine, NAP 226-90, 50 ng/mL [$^{2}H_{7}$]-donepezil, [^{13}C,$^{2}H_{3}$]-galantamine, [$^{13}C_{2}$,$^{2}H_{6}$]-memantine and 15 ng/mL [$^{2}H_{6}$]-rivastigmine).

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 $100 \pm 15\%$ (LLOQ $\pm 20\%$) with values comprised between 86% and 108%. Moreover, the values for repeatability and intermediate precision met the requirements of CVs $\leq 15\%$ (LLOQ $\leq 20\%$) 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

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

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 ($\beta = 90\%$) are presented in Fig. 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

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)

	Rivastigmine ($n = 5$)		NAP 226-90 ($n = 5$)	
	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)

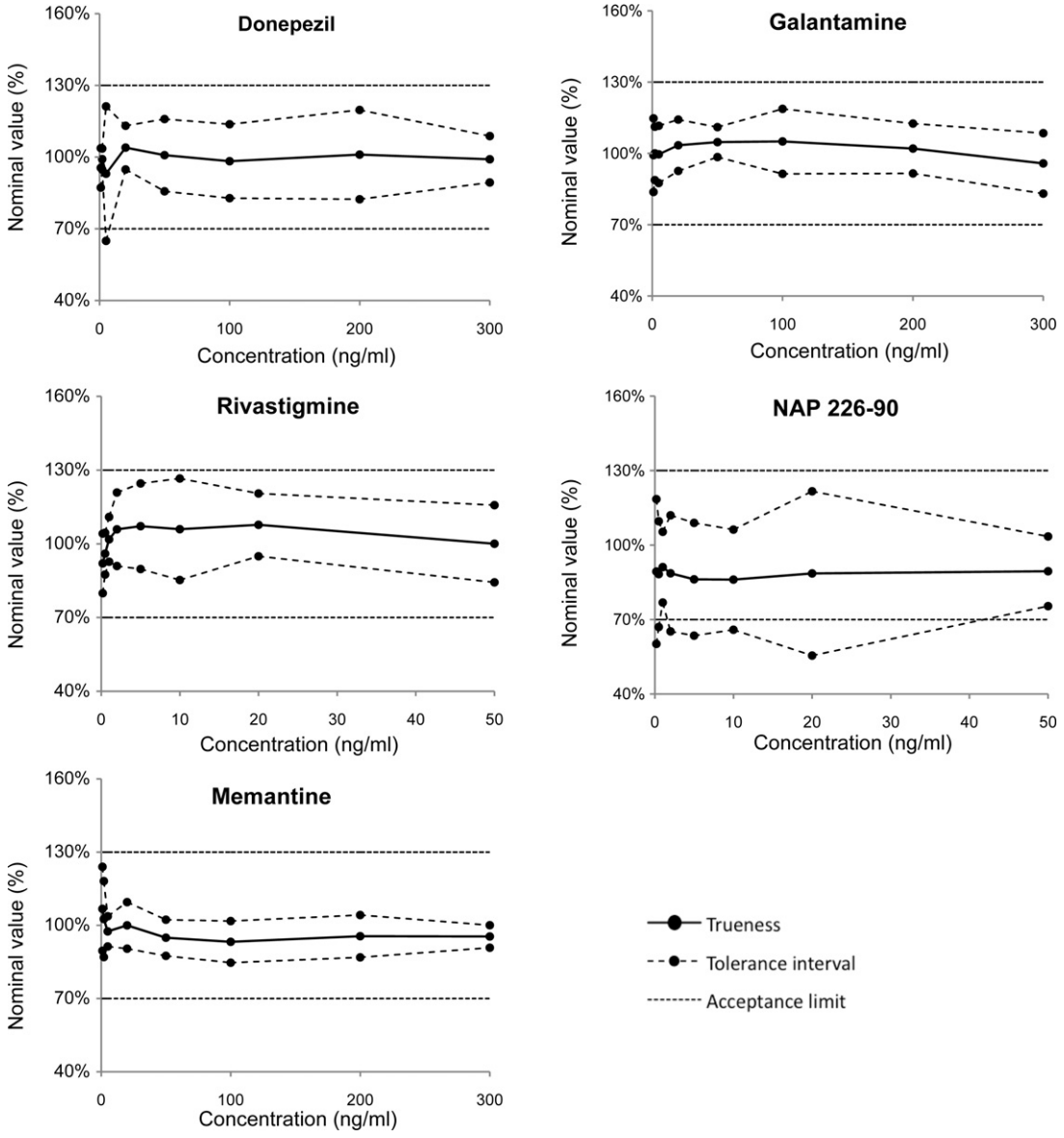

Fig. 3. Accuracy profiles within the acceptance limit ($\lambda = \pm 30\%$) and with upper and lower β-expectation tolerance intervals ($\beta = 90\%$) for each compound in the dosing range.

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 plasma 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 Fig. 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

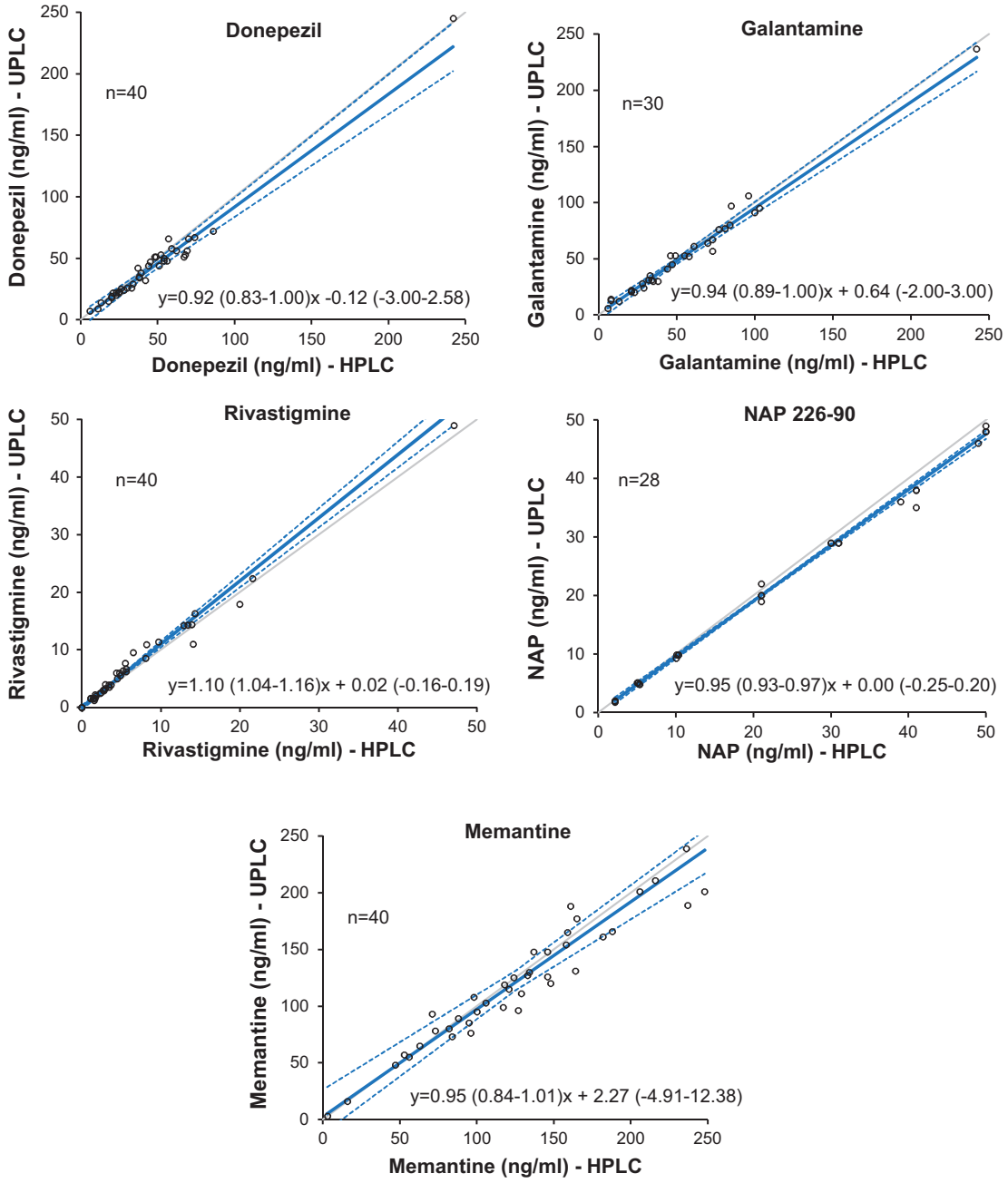

Fig. 4. Passing–Bablok fits of the comparison between UPLC–MS/MS and HPLC–MS. In the plots the regression lines (solid line), the 95% confidence intervals for the regression line (dashed lines, values in brackets) and the identity lines ($x = y$, gray line) are shown.

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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