

RESEARCH ARTICLE

A simple and direct HPLC-DAD method for the simultaneous determination of galantamine, donepezil and rivastigmine in cerebrospinal fluid, blood serum and urine

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Galantamine (GAL), donepezil (DON) and rivastigmine (RIV) are cholinesterase inhibitors administered to patients who suffer from Alzheimer's disease (AD). We have currently developed and validated an HPLC-DAD method for the determination of GAL, DON, RIV in cerebrospinal fluid (CSF), blood serum and urine. The retention times of the drugs were 1.5, 2.2 and 3.1 min, respectively and the total time of analysis was 5 min. Validation was performed in terms of linearity, selectivity, accuracy, precision and stability. Following validation, the method was applied to clinical CSF, blood serum and urine samples. The currently developed method is a reliable tool for monitoring CSF, serum and urine galantamine, donepezil and rivastigmine levels in patients under treatment with these drugs.

Keywords: cholinesterase inhibitors, cerebrospinal fluid, blood serum, urine, HPLC, diode array detection.

Introduction

Galantamine, donepezil and rivastigmine are drugs prescribed in Alzheimer's Disease (AD). Galantamine hydrobromide, donepezil hydrochloride and rivastigmine are inhibitors of acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), which hydrolyze the neurotransmitter acetylcholine. Therefore, galantamine hydrobromide, donepezil hydrochloride and rivastigmine exert their therapeutic effect by increasing the concentration of acetylcholine and enhancing the cholinergic function [1]. It has been shown that AChE and BuChE inhibition in CSF correlates positively with cognitive performance [2]. Furthermore, the monitoring of the enzyme's activity and the drug concentration in CSF is of clinical impor-

tance according to the latest scientific data[3,4]. Thus, the availability of a reliable analytical technique for the determination of these drugs in CSF, blood serum and urine is of utmost importance both in the clinical, as well as in the forensic toxicology setting.

A number of methods have been developed until today for the separate determination of galantamine, donepezil and rivastigmine in plasma [5-34]. Additionally, a few methods have been reported for the separate determination of these drugs in serum and urine [35-39]. In most of the aforementioned cases, High Pressure Liquid Chromatography, coupled with mass spectrometry or tandem mass spectrometry, was used. UV and fluorescence have also been used for detection. The preparation techniques used were mainly solid phase extraction, liquid-liquid extraction and protein precipitation.

To the best of our knowledge, a fully validated method for the determination of donepezil in CSF has only

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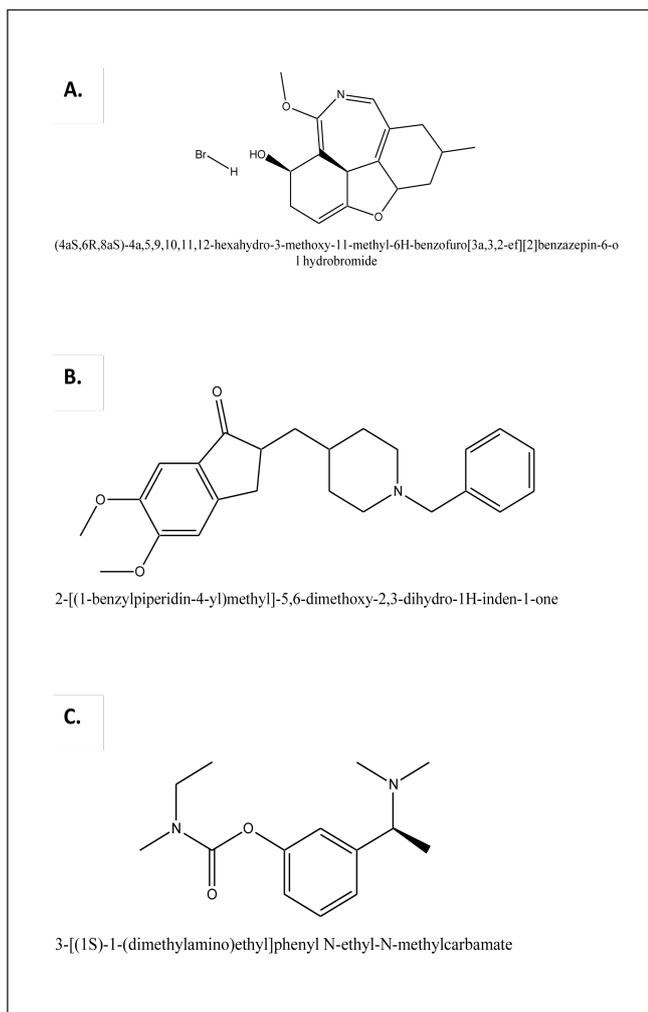


Figure 1. Chemical structures of cholinesterase inhibitors: A) galantamine, B) donepezil, C) rivastigmine.

been reported once until today [40]. Furthermore, only two methods have been developed for the simultaneous detection of four antedementia drugs (donepezil, galantamine, rivastigmine and memantine) in human plasma by LC-MS and UPLC-MS/MS [41,42]. Thus, a validated method for the simultaneous determination of galantamine, donepezil and rivastigmine in CSF, blood serum and urine has not been reported until today. For this reason, we have developed and validated a simple HPLC method, with a use of a diode-array detector, for the determination of these particular AChE and BuChE inhibitors in CSF, blood serum and urine. This method will be further employed into clinical practice in order to assess a possible correlation between the CSF levels of ChE inhibitors, AChE and BuChE activity in CSF and clinical outcome in patients with cognitive impairment. The chemical structures of the drugs of interest used are shown in **Figure 1**.

Materials and Methods

Chemicals and Reagents

Acetic acid and sodium acetate trihydrate were purchased from Merck (Darmstadt, Germany). Donepezil hydrochloride was a kind donation from the pharmaceutical company Elpen. Rivastigmine (purity 99,9%) was purchased from the pharmaceutical company Carbosynth (Compton, Berkshire, UK) and galantamine was derived from its pharmaceutical formulation which is commercially available in Greece (Galantamine hydrobromide, 8 mg/capsule, Generics Pharma Hellas, a subsidiary of Mylan INC).

All organic solvents (acetonitrile, methanol), as well as water, were of HPLC grade. Acetonitrile was purchased from LAB-SCAN, Analytical Sciences (POCH S.A., Gliwice, Poland). Methanol was purchased from Chromasolv (Fluka Analytical, Sigma-Aldrich, USA). Water was purchased from Chromasolv®Plus (Sigma-Aldrich, USA). Ammonia was purchased from Panreac Quimica S.A. (Barcelona, Spain).

Colchicine, tolfenamic acid, chlortetracycline, chloramphenicol and thiamphenicol were purchased from Sigma-Aldrich (PA, USA). Lamotrigine, sulfamethazine, sulfisoxazole, sulfadiazine, sulfaquinoxaline and sulfamethizole were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). Nalidixic acid and flumequine were purchased from Sigma (Steinheim, Germany). Oxolinic acid was purchased from Fluka (Buchs, Switzerland). Epichlorotetracycline was purchased from Fisher Scientific (Germany). Bamifyline was purchased from LGM Pharma, USA. Doxycycline was purchased from Oracea (USA). The above compounds were all evaluated as possible internal standards.

Preparation of standards

Stock standard solutions of the examined drugs (160 ng/ μ L for galantamine, 1000 ng/ μ L for donepezil and rivastigmine) were prepared in methanol, with the exception of donepezil, for which the stock standard solution was prepared in water, due to the better solubility of the drug in this solvent. All standard solutions were stored at 4°C.

From the above stock standard solutions, eight aquatic working standards were prepared in the range of 0.5–40 ng/ μ L. Calibration standards of the cholinergic drugs in the three different biological matrices were prepared by spiking drug-free samples with standard solutions.

Instrumentation and Chromatography

Analysis was performed on an UHPLC system which

consisted of a Shimadzu Prominence liquid chromatograph with a 10 μL injection loop, coupled with a Shimadzu SPD-M20A Diode Array UV detector (Kyoto, Japan). The analysis was carried out on an ODS Hyper-sil column (C18 classical, 250 \times 2,1 mm, 5 μm) (Thermo Fisher Scientific, Germany). The mobile phase consisted of Solvent A: acetonitrile, Solvent B: methanol and Solvent C: buffer solution of sodium acetate/acetic acid, 0.2M, pH 4.8. The time program used was: 0 min-solvent A 45% (v/v), solvent B 20% (v/v) and solvent C 35% (v/v) – flow rate at 0.6 mL/min, 3 min-solvent A 45% (v/v), solvent B 45% (v/v) and solvent C 10% (v/v) – flow rate at 0.9 mL/min. The maximum inlet pressure was 3800 psi. Analysis was completed within 5 min. UV detection was performed at 215 nm (rivastigmine), 232 nm (donepezil) and 290 nm (galantamine). Data acquisition was performed by the LCsolution, version 1.25 (Shimadzu Corporation) software.

Sample collection

Post-mortem, drug-free CSF samples were collected, as part of the standard operating procedure, upon autopsy at the Forensic Medical Service of Thessaloniki, Greece. Blood serum and urine were collected from healthy volunteers, who refrained from using any medication. Clinical CSF, blood and urine samples were collected from patients under treatment with cholinergic drugs, following written informed consent.

Sample preparation

The CSF samples (50 μL) were subjected to protein precipitation with the addition of 100 μL of CH_3CN . Following centrifugation, the supernatant was evaporated (not to dryness) under a gentle stream of nitrogen and was then loaded onto the pre-conditioned SPE cartridge. The blood serum samples (50 μL) were subjected to protein precipitation with the addition of 400 μL CH_3CN . Following centrifugation, the supernatant was evaporated (not to dryness) under a gentle stream of nitrogen and was then loaded onto the pre-conditioned SPE cartridge. The urine samples (50 μL) were treated in the same way as the CSF samples and were then loaded onto the pre-conditioned SPE cartridge.

Solid phase extraction procedure

For all biological matrices, the cartridges were initially conditioned with 2 mL CH_3OH and equilibrated with 2 mL H_2O . The sample was then loaded onto the cartridge StrataTM-X-Drug B 33 μm Polymeric Strong Cation [30 mg/3 mL, Phenomenex[®] (USA)]. The cartridge was washed with 500 μL H_2O and the drugs were eluted with

1 mL CH_3OH (containing 5% ammonia). The eluent was evaporated to dryness under a gentle stream of nitrogen. It was then reconstituted with 200 μL of CH_3OH and 10 μL were injected onto the HPLC column.

Method Validation

Linearity

Linear regression analysis was used to determine linearity. For this purpose, working standard solutions and spiked CSF, blood serum and urine samples, containing cholinergic drugs at different concentrations, within the range of 0.5–40 ng/ μL for donepezil and galantamine and 5–40 ng/ μL for rivastigmine, were used. The peak area ratio of the analyte was plotted versus the nominal drug concentration in order to construct the calibration curve. Least square linear regression analysis was used to calculate the slope, intercept, and coefficient of determination. The formula $\text{LOD} = 3.3 S/N$ was used for the calculation of the limit of detection (LOD) and the formula $\text{LOQ} = 10 S/N$ was used for the calculation of the limit of quantitation (LOQ), where S= signal, and N= noise.

Recovery and Precision/Accuracy

Mean absolute recovery of galantamine was evaluated by comparing spiked CSF, blood serum and urine samples to standard solutions. It was found to be 96%, 85% and 96%, respectively in the three biological matrices. Similarly, mean absolute recovery of donepezil was found to be 96% in CSF, 99% in blood serum and 97% in urine. Mean absolute recovery of rivastigmine was found to be 86% in CSF, 76% in blood serum and 81% in urine.

In order to evaluate the precision and accuracy of the developed method, CSF, blood serum and urine samples, spiked at different concentration levels (in the range 0.5–40 ng/ μL for donepezil and galantamine and 5–40 ng/ μL for rivastigmine), were analyzed. The respective linear regression equation was always used to calculate the nominal contents of the drug. The results were expressed as percent recoveries (mean found concentration/theoretical concentration)/100. The within-day RSD was used as a measure of the repeatability of the method, while the SD between nominal and measured concentrations was used to express precision. CSF, blood serum and urine samples spiked at four different concentrations were analyzed multiple times in order to determine within-day and between-day precision and accuracy. Four measurements performed on the same day were used to assess within-day repeatability. Furthermore, four measurements at four consecutive days were used to evaluate between-day repeatability.

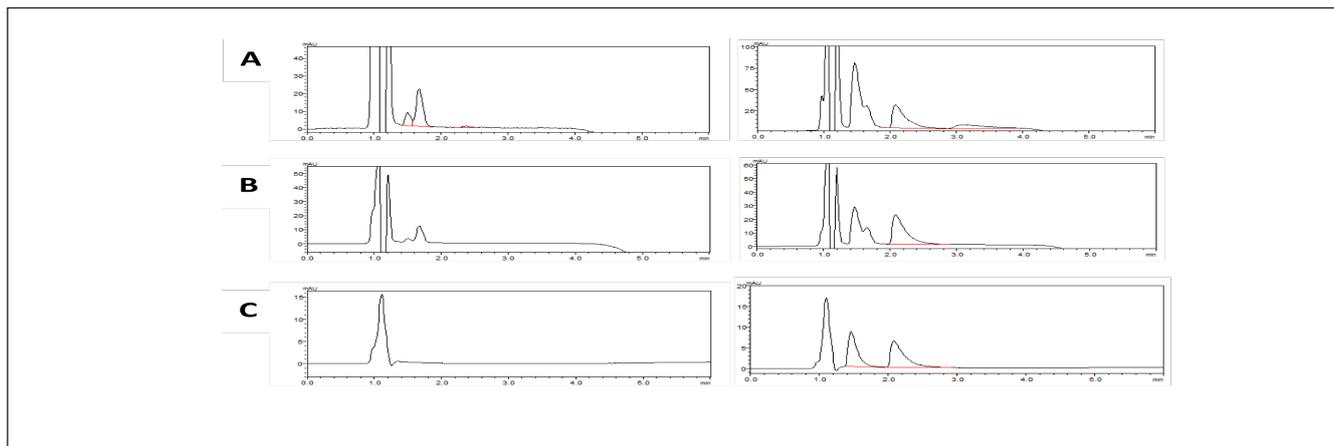


Figure 2. Typical HPLC chromatogram of a blank CSF (left) and spiked CSF sample (15 ng/μL) (right): A) 215 nm, B) 232 nm, C) 290 nm. Conditions are described in text.

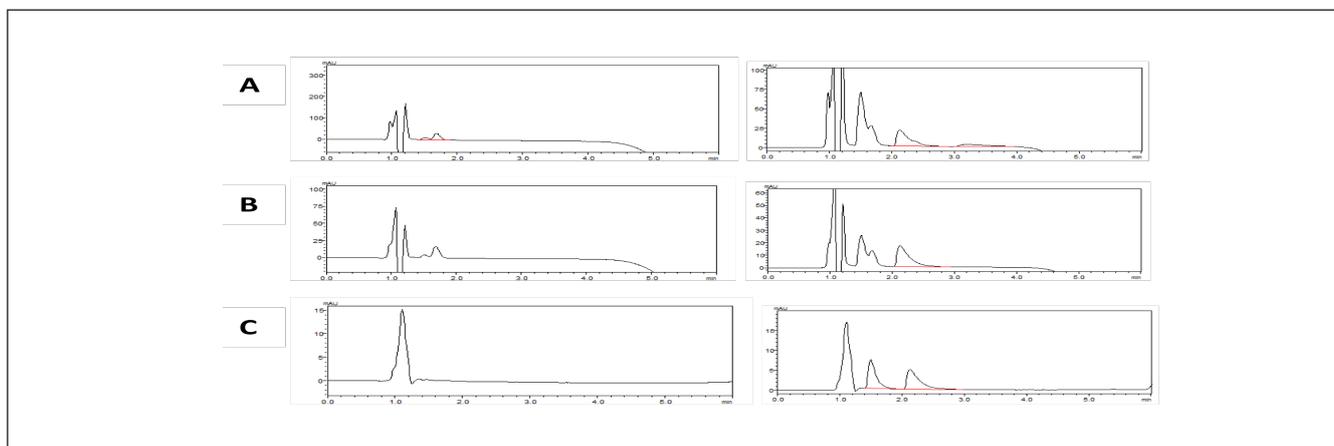


Figure 3. Typical HPLC chromatogram of a blank blood serum (left) and spiked serum sample (15 ng/μL) (right): A) 215 nm, B) 232 nm, C) 290 nm. Conditions are described in text.

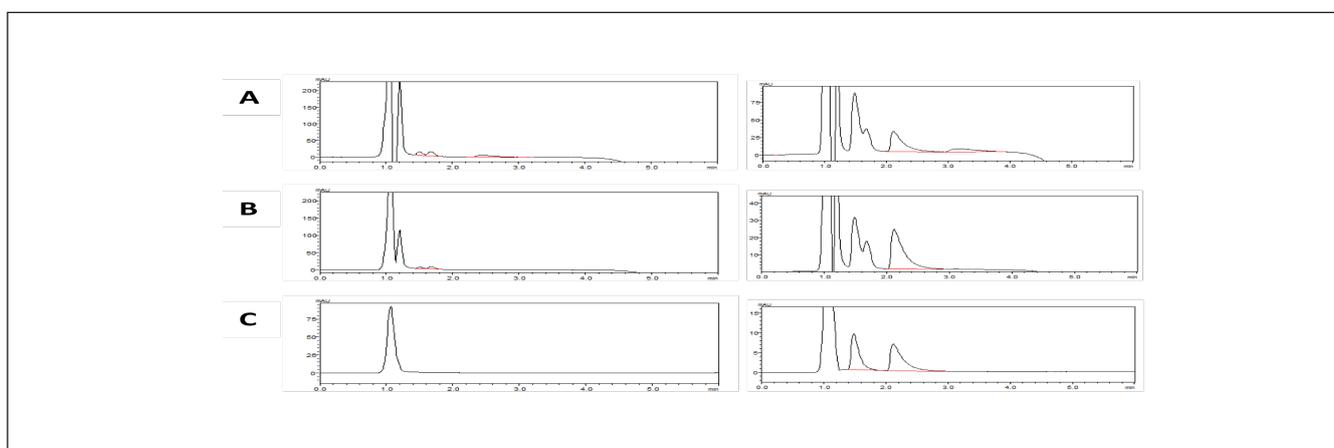


Figure 4. Typical HPLC chromatogram of a blank urine (left) and spiked urine sample (15 ng/μL) (right): A) 215 nm, B) 232 nm, C) 290 nm. Conditions are described in text.

Table 1. Calibration and sensitivity data of the examined cholinergic drugs in aqueous standard solutions, CSF, blood serum and urine samples.			
Aqueous standard solutions			
	GAL	DON	RIV
Regression data*	$Y=(6131.30 \pm 82.35) X + (-915.27 \pm 1413.02)$	$Y=(13277.65 \pm 435.29) X + (-6420.86 \pm 7468.84)$	$Y=(10850.48 \pm 851.72) X + (-24516.33 \pm 18464.92)$
R	0.9989	0.9936	0.9819
LOD (ng/ μ L)	0.8	1.9	5.6
LOQ (ng/ μ L)	2.3	5.6	17.0
CSF			
Regression data	$Y=(5948.17 \pm 229.89) X + (2012.29 \pm 3944.43)$	$Y=(21964.39 \pm 1163.01) X + (15449.66 \pm 19955.27)$	$Y=(6204.9 \pm 395.8) X + (26812.7 \pm 8580.4)$
R	0.9911	0.9835	0.9879
LOD (ng/ μ L)	2.2	3.0	4.6
LOQ (ng/ μ L)	6.6	9.1	13.8
Bloodserum			
Regression data	$Y=(4886.57 \pm 239.36) X + (-317.78 \pm 4106.98)$	$Y=(18855.8 \pm 1040.0) X + (-9628.19 \pm 17844.62)$	$Y=(6204.9 \pm 395.8) X + (26812.7 \pm 8580.4)$
R	0.9858	0.9821	0.9879
LOD (ng/ μ L)	2.8	3.1	4.6
LOQ (ng/ μ L)	8.4	9.5	13.8
Urine			
Regression data	$Y=(6109.89 \pm 227.35) X + (-3878.7 \pm 3901.0)$	$Y=(23306 \pm 931) X + (14552.1 \pm 15974.4)$	$Y=(6031.26 \pm 158.25) X + (11559.74 \pm 3430.67)$
R	0.9918	0.9905	0.9979
LOD (ng/ μ L)	2.1	2.3	1.9
LOQ (ng/ μ L)	6.4	6.8	5.7
*Y= peak area X= ng/ μ L; Linearity range: 0.5-40 ng/ μ L for galantamine, donepezil and 5-40 ng/ μ L for rivastigmine			

Selectivity

The selectivity of the developed method was demonstrated by the absence of interference in the chromatographic window of interest from degradation products and endogenous matrix components. The selectivity of the method was evaluated by analyzing drug-free CSF, blood serum and urine samples.

Stability

As part of the validation process, we evaluated both the

long- and the short- term stability of galantamine, donepezil and rivastigmine in CSF, blood serum and urine. For this purpose, drug-free CSF, blood serum and urine samples were spiked with the three drugs at a final concentration of 15 ng/ μ L. The stability of cholinergic drugs was assessed under different storage conditions (at 25°C for 1, 2 and 24 h, at 4°C for 1, 2, 3 days, and at -24°C for 1, 2, 4, 6 weeks). The stability of galantamine, donepezil and rivastigmine was also evaluated during the repetitive freezing and thawing of CSF, blood serum and urine. For

Table 2. Within-day repeatability, between-day precision and accuracy for the determination of the examined cholinergic drugs in CSF, blood serum and urine.

CSF						
Added (ng/ μ L)	GAL			Between-day (n=4x4)		
	With-in day (n=4)		Recovery (%)	Found \pm SD (ng/ μ L)	RSD	Recovery (%)
	Found \pm SD (ng/ μ L)	RSD				
1	1.06 \pm 0.03	2.8	106	1.09 \pm 0.09	8.2	109
5	3.83 \pm 0.04	1	77	3.7 \pm 0.3	7	74
15	11.4 \pm 0.5	4.3	79	11.4 \pm 0.6	5.6	76
20	18.6 \pm 0.7	3.9	93	18.0 \pm 0.9	5.1	90
DON						
1	1.07 \pm 0.09	8.4	107	1.1 \pm 0.5	4.3	108
5	3.75 \pm 0.05	1.2	75	3.6 \pm 0.1	3.5	72
15	12.1 \pm 0.5	4.1	80	12.1 \pm 0.4	3.7	80
20	18.2 \pm 0.6	3.1	91	17.9 \pm 0.8	4.3	89
RIV						
5	4.1 \pm 0.2	5.1	82	4.2 \pm 0.4	8.9	85
15	15.0 \pm 1.1	6.8	100	15.7 \pm 1.1	7.2	105
20	22.6 \pm 0.4	1.9	113	20.8 \pm 1.6	7.6	104
Blood serum						
GAL						
1	1.05 \pm 0.06	5.7	108	1.08 \pm 0.07	6.5	108
5	4.2 \pm 0.2	3.6	83	4.2 \pm 0.3	6.8	84
15	11.65 \pm 0.09	0.7	78	11.4 \pm 0.6	5.7	76
20	17.8 \pm 0.2	0.9	89	17.2 \pm 0.8	4.9	86
DON						
1	1.10 \pm 0.06	5.4	110	1.02 \pm 0.09	8.8	102
5	4.6 \pm 0.2	4.2	92	4.6 \pm 0.1	2.9	93
15	12.7 \pm 0.2	1.7	85	12.5 \pm 0.4	3.3	83
20	19.3 \pm 0.1	0.7	96	18.6 \pm 0.6	3.3	93
RIV						
5	5.4 \pm 0.3	5.0	108	5.4 \pm 0.3	6.0	107
15	12.8 \pm 0.1	1.1	85	12.8 \pm 0.1	1.0	86
20	17.2 \pm 0.1	2.3	86	17.3 \pm 0.5	3.0	86
Urine						
GAL						
1	1.06 \pm 0.02	1.9	106	1.1 \pm 0.1	104	106
5	4.14 \pm 0.05	1.3	83	4.0 \pm 0.2	5.7	80
15	13.0 \pm 0.2	1.4	87	12.6 \pm 0.6	4.5	84
20	17.8 \pm 0.1	0.6	89	17.1 \pm 0.6	3.8	85
DON						
1	1.06 \pm 0.05	4.7	106	1.1 \pm 0.1	10.4	106
5	4.06 \pm 0.06	1.6	81	4.0 \pm 0.2	5.7	80
15	14.1 \pm 0.1	0.8	94	12.6 \pm 0.6	4.5	84
20	18.9 \pm 0.4	1.9	94	17.1 \pm 0.6	3.8	85
RIV						
5	5.5 \pm 0.5	9.1	110	4.3 \pm 0.5	11.6	86
15	16.5 \pm 0.9	5.2	110	16.9 \pm 1.8	10.6	113
20	20.3 \pm 0.2	0.7	101	22.0 \pm 1.9	8.5	110

this purpose, the 10% degradation criterion was used to evaluate stability.

Results and discussion

Typical chromatograms of drug-free (blank) CSF, blood serum and urine samples as well as typical chromatograms of spiked CSF, blood serum and urine samples are shown in **Figures 2, 3 and 4** respectively.

The retention time for galantamine, donepezil and rivastigmine was 1.5, 2.2 and 3.1 min, respectively.

The linearity was evaluated in the 0.5-40 ng/ μ L range for galantamine and donepezil and 5-40 ng/ μ L for rivastigmine. Linear-regression analysis was applied. Linearity and sensitivity data are summarized in **Table 1**.

The accuracy (assessed by means of recovery percentage), was evaluated by the determination of galantamine, donepezil and rivastigmine in spiked CSF, blood serum and urine samples at four concentration levels. Repeatability was evaluated by analyzing samples spiked with the analyte at four concentration levels, in four replicates. Intermediate precision was assessed by the between-day variation over a period of four days. Accuracy and precision data are summarized in **Table 2**.

Selectivity (absence of interference from the sample matrix in the same chromatographic window as the examined drugs) was evaluated by the analysis of blank CSF, blood serum and urine samples. As shown in **Figure 2** (blank CSF sample), **Figure 3** (blank blood serum sample) and **Figure 4** (blank urine sample) there was no interference in the analysis of the selected drugs from the particular biological matrices examined.

To examine the stability of the analytes, drug-free CSF, blood serum and urine samples were spiked with galantamine, donepezil and rivastigmine at a final concentration of 15 ng/ μ L. Stability was assessed following storage at ambient temperature (25°C) for 1, 2 and 24 h, at 4°C for 1, 2 and 3 days, and at -24°C for 1, 2, 4 and 6 weeks.

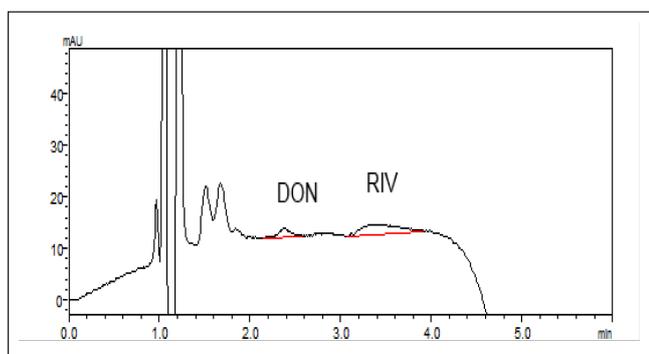


Figure 5. Typical HPLC chromatogram of a clinical CSF sample (CSF 76) at 215 nm. Conditions are described in text.

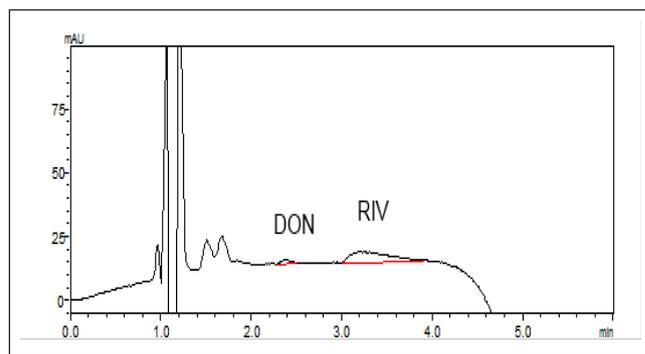


Figure 6. Typical HPLC chromatogram of a clinical blood serum sample (SERUM 1956) at 215 nm. Conditions are described in text.

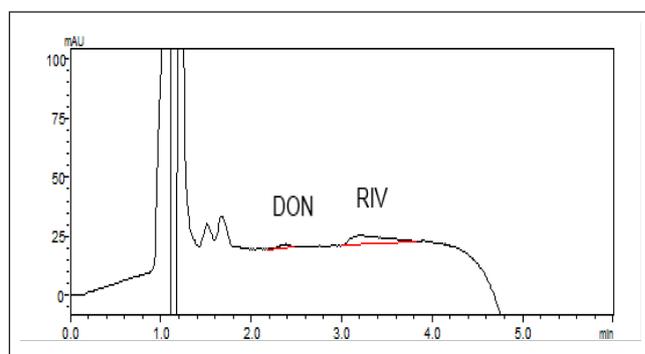


Figure 7. Typical HPLC chromatogram of a clinical urine sample (URINE 1956) at 215 nm. Conditions are described in text.

Table 3. Concentration of donepezil and rivastigmine in clinical CSF, blood serum and urine samples obtained from different patients under donepezil and rivastigmine treatment.

Sample ID	C _{donepezil} (ng/ μ L)	C _{rivastigmine} (ng/ μ L)
CSF 104	1.1	-
CSF 92	0.1	-
CSF 76	0.02	0.2
CSF 81	-	4.3
Serum 1956	0.8	0.2
Serum 1935	1.1	-
Serum 1938	-	0.8
Serum 1944	0.3	-
Urine 1956	0.8	0.1
Urine 1935	0.7	-
Urine 1938	-	5.0
Urine 1944	1.2	-

During storage of spiked CSF samples at -24°C , galantamine remained stable for 4 weeks, donepezil for 2 weeks and rivastigmine for 1 week. During storage at 4°C , galantamine and donepezil remained stable for 3 days, while rivastigmine remained stable only for 1 day. During storage at ambient temperature (25°C) all three drugs remained stable for 2 hours (h). To examine the stability of galantamine, donepezil and rivastigmine in CSF during repetitive freeze-thaw cycles (from -24°C to room temperature), drug-free CSF was spiked at a final concentration of $15\text{ ng}/\mu\text{L}$ for galantamine, donepezil and rivastigmine. In CSF, galantamine remained stable for 2 cycles, donepezil for 4 cycles and rivastigmine for 1 cycle. Degradation was evaluated using the 10% criterion. During storage of spiked blood serum samples at -24°C , all three drugs remained stable for 4 weeks. During storage at 4°C , all three drugs remained stable for 3 days. During storage at ambient temperature (25°C) all three drugs remained stable for 2 hours (h). To examine the stability of galantamine, donepezil and rivastigmine in blood serum during repetitive freeze-thaw cycles (from -24°C to room temperature), drug-free blood serum was spiked at a final concentration of $15\text{ ng}/\mu\text{L}$ for galantamine, donepezil and rivastigmine. In blood serum, all three drugs remained stable for 1 cycle. Degradation was evaluated using the 10% criterion. During storage of spiked urine samples at -24°C , all three drugs remained stable for 4 weeks. During storage at 4°C , donepezil and galantamine remained stable for 3 days, while rivastigmine remained stable for 1 day. During storage at ambient temperature (25°C) galantamine and donepezil remained stable for 2 hours (h), while rivastigmine remained stable only for 1 hour. To examine the stability of galantamine, donepezil and rivastigmine in urine during repetitive freeze-thaw cycles (from -24°C to room temperature), drug-free urine was spiked at a final concentration of $15\text{ ng}/\mu\text{L}$ for galantamine, donepezil and rivastigmine. In urine, galantamine remained stable for 2 cycles, donepezil for 6 cycles and rivastigmine for 4 cycles. Degradation was evaluated using the 10% criterion.

Application of the developed method to clinical CSF, blood serum and urine samples

The developed method was applied to clinical CSF, blood serum and urine samples obtained from patients under treatment with donepezil and rivastigmine.

Typical chromatograms of clinical CSF, blood serum and urine samples are shown in **Figures 5, 6 and 7**, respectively.

The standard addition method was applied to some sam-

ples where donepezil and rivastigmine concentration was below the LLOQ of the method.

Table 3 shows the concentrations of donepezil and rivastigmine in the different clinical CSF, blood serum and urine samples analyzed.

Conclusions

The degree of AChE and BuChE inhibition in CSF, by AChE and BuChE inhibitors (such as donepezil, galantamine and rivastigmine) is of clinical importance in patients with cognitive impairment and is possibly associated with the concentration of the inhibitor in CSF.

There are currently two validated methods for the simultaneous determination of galantamine, donepezil and rivastigmine by HPLC-MS/MS and UHPLC-MS/MS, respectively. Because mass detection demands expensive instrumentation, we have currently developed and validated a fast and simple HPLC-DAD method for the simultaneous determination of three cholinergic drugs in CSF, blood serum and urine. The proposed method was validated in terms of linearity, selectivity, accuracy, precision and stability and proved to be robust and reliable. The developed method will be further employed in order to assess a possible correlation between the CSF levels of ChE inhibitors, AChE and BuChE activity in CSF and clinical outcome in patients with cognitive impairment. This method can also be applied in the clinical toxicology setting, as well as in the forensic toxicology setting and to the best of our knowledge, it is the only available method for the simultaneous determination of these drugs in CSF, urine and blood serum by a simple HPLC-DAD method.

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