METHOD DEVELOPMENT FOR THE ANALYSIS OF PHARMACEUTICALS WITH ACETHYLCHOLINESTERASE ACTIVITY IN WATER USING HPLC-DAD AND SOLID PHASE EXTRACTION

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ABSTRACT

An SPE followed by HPLC-DAD method with ion pair chromatography technique to analyze pharmaceuticals with acethylcholinesterase activity including pyridostigmine (PYR), galathamine (GAL), neostigmine (NEO), eserine (ESE), and donepezil (DON) in water samples was developed. Acetylcholinesterase (AChE) inhibitors have been used to treat less severe dementias such as Alzheimer's disease. Chromatographic separation was achieved using reversed-phase SymmetryShield column using gradient system with mobile phase consisting of H_2O/ACN (99:1, v/v) as mobile phase A with 10 mM sodium 1-hexanesulfonate and 0.1% acetic acid (HAc). The HPLC/DAD method was linear between concentrations of 5 to 100 ng/µL. The IDL and IQL ranged from 0.50 to 1.25 ng/µL and 1.5 to 3.0 ng/µL, respectively. SPE was used to extract and clean up the target substances in spiked pure water, tap water, and wastewater samples. The application of extraction method of 5 target substances in wastewater sample was divided into 2 parts: Oasis WCX (6 mL, 500 mg) for PYR and Oasis HLB (6 mL, 200 mg) for GAL, NEO, ESE and DON. The developed SPE and HPLC/DAD method is applicable for quantification of the 5 target substances in water samples in a concentration range > 50 µg/L and assumable lower for DON (> 25 µg/L).

Keywords: *acetylcholinesterase; solid phase extraction; ion pair chromatography*

ABSTRAK

Dalam penelitian ini telah dikembangkan metode SPE diikuti HPLC-DAD dengan tehnik kromatografi pasangan ion untuk menganalisis lima senyawa yang memiliki aktivitas acethylcholinesterase yaitu pyridostigmine (PYR), galathamine (GAL), neostigmine (NEO), eserine (ESE) dan donepezil (DON) pada sampel air. Inhibitor asetilkolinesterase (AChE) telah digunakan untuk mengobati penyakit dementia seperti Alzheimer. Pemisahan kromatografi diperoleh dengan menggunakan kolom fasa terbalik, SymmetryShield dan menggunakan sistem gradien dengan fasa gerak terdiri dari H_2O/ACN (99:1, v/v) yang mengandung 10 mM natrium 1-hexanasulfonat dan 0,1% asam asetat (HAc). Metode HPLC/DAD menunjukkan linearitas antara konsentrasi 5 sampai dengan 100 ng/µL. Nilai IDL diperoleh diantara 0,50 dan 1,25 ng/µL, sedang nilai IQL diantara 1,5 dan 3,0 ng/µL. SPE digunakan untuk ekstraksi dan pencucian 5 senyawa target pada sampel air murni, air kran dan air limbah yang telah ditambahkan larutan standar dengan konsentrasi tertentu. Metode ekstraksi untuk kelima senyawa target pada sampel air limbah dibagi menjadi 2 bagian dimana Oasis WCX (6 mL, 500 mg) digunakan untuk mengektraksi PYR dan Oasis HLB (6 mL, 200 mg) untuk GAL, NEO, ESE dan DON. Pengembangan metode SPE diikuti HPLC-DAD dapat digunakan untuk melakukan kuantifikasi 5 senyawa target pada sampel air dengan konsentrasi > 50 µg/L dan lebih rendah untuk DON (> 25 µg/L).

Kata Kunci: *asetilkolinesterase; solid phase extraction; kromatografi ion pair*

INTRODUCTION

Pharmaceuticals in the aquatic environment have been recognized as one of the emerging issues in environmental chemistry in the recent years [1]. Due to high polarity and persistence of these active substances, many of them are only slightly transformed or even unchanged thus could contaminate the

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receiving water and environment. Various pharmaceuticals and its metabolites are found nowadays in surface waters, underground waters and even drinking waters as well.

Acetylcholinesterase (AChE) inhibitors have been used in the treatment of human diseases, the control of insect pests, and even as chemical warfare agents. Some inhibitors are used as antidementias such as Alzheimer's disease because they have beneficial effect on cognitive functions for patients with less severe forms of the disease. The organization, Alzheimer's Disease International [2], reported that there are an estimated 35.6 million people which suffer from dementia worldwide by 2010. These numbers will nearly double every 20 years, thus 65.7 million are estimated for 2030 and 115.4 million for 2050. Since the Alzheimer's disease patients tend to increase in number, there is indication of increasing usage of antidementia drugs especially in the ageing population and consequently may also cause wastewater pollutions.

In order to anticipate the drawback effect of the presence of these AChE inhibitor pharmaceutical in environment, a controlling and monitoring must be done continuously in every water compartment in environment including the wastewater, surface, groundwater, and even drinking water. An analytical method with the high sensitivity and selectivity is needed to detect and to monitor the presence of ACh inhibitors simultaneously in water compartment in environment. In addition, extraction method for AChE inhibitor substances from the water matrix should be developed. The chemical structures, physical and chemical properties of the five AChE inhibitor drugs are presented in Table 1. All of the studied AChE inhibitors are moderate to strong basic compounds with pK_a values that range between 6.1 and 12.2. The lipophilicities of the 5 compounds are characterized by log K_{ow} values which range from 0.9 to 4.9. All of the substances are easily soluble in water with solubilities greater than 1000 mg/L.

Many methods for the determination and quantification of of AChE inhibitors in biological and drugs samples were reported in literature particularly with chromatographic techniques like HPLC with UV [4-6], fluorescence [7], DAD [8] or MS/MS detection [9]. For single substance measurement, HPLC method with UV detector provided good sensitivity and selectivity for the estimation of DON in tablets with the concentration range 2–60 µg/mL [10]. HPLC method with DAD detector gives not only good sensitivity and selectivity but also applicable for simultaneously measurement.

Sample preparation of human serum for HPLC analysis of AChE inhibitors was performed by applying ion pair LLE extraction method using picric acid as ion pair reagent for isolating PYR [11]. Comparison study between LLE and SPE of GAL from blood plasma and tissues by Maláková et al. [12] revealed that extraction with Oasis MCX cartridges provided an acceptable extraction recovery of 81.0% and cleaner samples for the separation on chromatographic column compare to LLE. Schonberg et al. [13] compared hydrophobic, weak (WCX) and strong cation-exchange (SCX) material to extract basic compounds including NEO from urine sample and only the polymer based WCX sorbent met the requirements of the method concerning sample clean-up and elution with mobile phase. Octadecyl (C18)-bonded silica such as C18 SEP PAK was the most widely applied SPE adsorbent to extract PYR [14]. Cherstniakova et al. [15] reported extraction of PYR in human plasma by using Oasis HLB cartridges.

The aim of this study was the development and validation of a chromatographic reference method using SPE and HPLC/DAD for the simultaneous detection of AChE inhibiting pharmaceuticals in water samples. In this study, spiked pure, tap and wastewater was used as model with increasing of matrices complexity. The AChE inhibitors PYR, GAL, NEO, ESE and DON were selected as target compounds as these substances are active agents of approved pharmaceuticals in Germany and reference standards of the single substances were commercially available.

EXPERIMENTAL SECTION

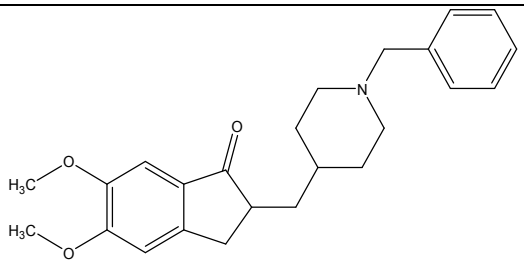
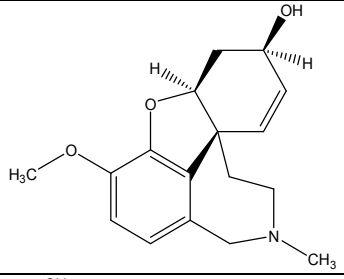
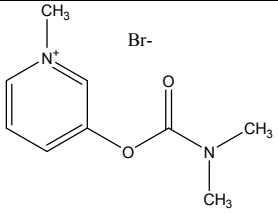
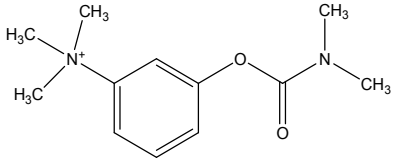
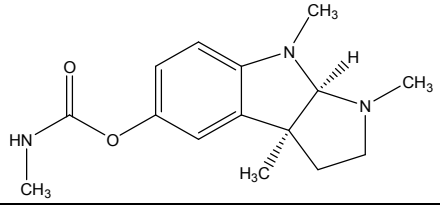
Materials

Pyridostigmine bromide, galanthamine hydrobromide (≥ 94%), neostigmine bromide (98%), donepezil hydrochloride monohydrate (≥ 98%) and eserine were purchased from Sigma-Aldrich. Sodium 1-hexanesulfonic acid as ion pair reagent was purchased from Sigma-Aldrich, acetonitrile was purchased from HiPerSolv, methanol (HPLC grade) from Fischer Scientific, NaOH and acetic acid from Carl Roth, HCl and ammonia 25% from AnalaR NORMAPUR. Pure water was prepared using SERALPUR PRO 90/PRO 90 C Ultrapure water-System with 0.2 µm filters from SERAL, tap water was taken from regular tap water in laboratory, influent wastewater samples were collected from the waste water treatment plant in Braunschweig, Germany. Glass fiber filter, 0.45 µm mesh MN GF – 6 Ø 150 mm was purchased from Macherey-Nagel. SPE cartridges C18 polar plus was purchased from J.T.Baker while Oasis HLB, Oasis MCX and Oasis WCX were purchased from Waters.

Instrumentation

HPLC/DAD analysis was performed using a system of a 1200 SL series HPLC including a vacuum

Table 1. Molecular structures, physical and chemical properties of the AChE inhibitors

Name	Molecular structure	MW	pK_a	Log K_{ow}	Solubility in water [mg/L]
Donepezil (DON)		415.96	8.83	4.9	12200
Galanthamine (GAL)		368.3	8.2	1.1	31000
Pyridostigmine bromide (PYR)		261.12	7.9	0.9	1040
Neostigmine (NEO)		303.2	12.0	2.1	1000
Eserine (ESE)		275.35	6.1 and 12.2	1.2	7760

degasser, a binary pump and an autosampler from Agilent technologies. Reversed-phase C18 column (SymmetryShield, 150 mm x 4.6 mm id, 3.5 µm particle size) and SymmetryShield pre-column (4 mm × 4 mm, 5 µm particle size) were purchased from Waters. pH was measured with a microprocessor pH Meter (pH 535 MultiCal, Weilheim, Germany) and pH-glass electrode SenTix61 (pH 0–14, 0–100 °C, 3 mol/L KCl) which was calibrated before measurement with standard buffer solutions at pH 4.00 ± 0.1, 7.00 ± 0.1 and 9.20 ± 0.1. Conductometer 340i was purchased from TetraCon. Ultrasonic bath Sonorex RK512S was purchased from Bandelin.

Procedure

Optimization of the HPLC conditions

In developing HPLC method, standard mixture solution containing 100 ng/µL of each analyte dissolved in methanol was directly injected to the HPLC-DAD. Due to characteristic of target compounds which were moderate to strong basic and polar compounds (pK_a range between 6.1 and 12.2) they had little or no retention on a reversed-phase stationary phase. In order to increase the retention time of these analytes, ion pair reagents ware added to the mobile phase as additives. In order to get base line separation of the target compounds, parameter such as pH, ion pair

Table 2. SPE procedures for the fortification experiments with C18 polar plus, Oasis HLB, Oasis MCX and Oasis WCX

	100 mL of pure water			
Sample pretreatment	100 µL NH_3 25%	-	100 µL 100% HAc	-
Spiking	500 µL of 100 ng/µL standard mixture solution (spiking level 500 µg/L)			
SPE phase	C18 polar plus (6 mL/500 mg)	Oasis HLB (3 mL/60 mg)	Oasis MCX (3 mL/60 mg)	Oasis WCX (6 mL/150 mg)
Conditioning	5 mL MeOH 5 mL pure water	3 mL MeOH 3 mL pure water	3 mL MeOH 3 mL pure water	5 mL MeOH 5 mL pure water
Percolation of sample	Flow rate 2 – 3 mL/min			
Washing of sample flask	2x5 mL pure water	2x3 mL pure water	2x3 mL pure water	2x5 mL pure water
Drying of cartridge	5 min			
Elution	10 mL MeOH	10 mL MeOH	10 mL of 5% NH_3 in MeOH	10 mL 2% HAc in MeOH
Evaporation	Using rotary evaporator to 2 mL followed by nitrogen stream			
Reconstitution	Reconstitution to 1 mL with MeOH			
HPLC/DAD analysis				

reagent and gradient system was optimized. A gradient system with acetonitrile (ACN)/water in different composition of water content for mobile phase A and ACN for mobile phase B was tested. For the determination of the target substances in the presence of matrix, in particular from wastewater samples, displaying the chromatograms at longer wavelengths 245, 270, and 315 nm was included. The injection volume was 10 µL, the column temperature 30 °C, and the flow rate 1 mL/min. The optimized conditions of HPLC-DAD that achieved base line separation is called first method. In wastewater sample, the PYR peak overlapped with a peak from matrix interferences. In order to separate the PYR peak from the interferences the conditions were further optimized. This optimal HPLC-DAD condition is called modified method.

Comparison of the recovery rates of four different SPE cartridges

The extraction of AChE inhibitors with four different SPE cartridges were compared under the optimized conditions using pure water as it causes no matrix interferences. The selected SPE procedures for the different cartridges are listed in Table 2. All fortification experiments were conducted using 100 mL pure water spiked with the mixed standard solution at a spiking level of 500 µg/L. The conditioning, loading and washing steps were nearly identical in each procedure, just differed in the solvent volume depending on the respective amount of the SPE phases. The solvent used in the elution steps differed depending on the type of SPE phase. The eluates were evaporated and reconstituted in 1 mL MeOH, respectively. The recovery rates from the fortification experiments with pure water were calculated by comparing the peak area of each target compound with the peak area of a 50 ng/µL mixed standard solution after HPLC analysis.

Determination of the capacity of SPE cartridges

In order to check the capacity and performance of Oasis WCX (6 mL, 500 mg) cartridges using a larger sample volume, a series of fortification experiments were conducted with 100 mL (n=4), 500 mL (n=2) and 1000 mL (n=2) tap water spiked with 500 µL of 100 ng/µL mixed standard solution, respectively. The cartridges were conditioned with 5 mL MeOH followed by 5 mL pure water pH 7.0 ± 0.1 adjusted with 1 M NaOH. The sample was passed through the cartridges at a flow rate of 2-3 mL/min. The sample flask was washed with 2x10 mL pure water of pH 7 and the cartridges were dried for 5 min. Analytes were eluted with 25 mL of 2% HAc in MeOH and the extract solution was concentrated under gentle nitrogen flow before reconstituted to 1 mL in MeOH.

Table 3. Sample preparation of tap and wastewater samples with Oasis HLB and Oasis WCX

	Extraction of PYR	Extraction of GAL, NEO, ESE and DON
Sample volume	100 mL tap water and wastewater sample	200 mL tap water and wastewater sample
SPE phase	Oasis WCX (6 mL, 500 mg)	Oasis HLB (6 mL, 200 mg)
Conditioning	5 mL methanol 5 mL pure water pH 7	5 mL methanol 5 mL pure water
Percolation of sample	Flow rate 2 – 3 mL/min	
Washing of sample flask	2x5 mL pure water pH 7	2x5 mL pure water
Washing step	-	15 of mL 10% methanol (in case of wastewater)
Drying	5 min	
Elution	25 mL 2% HAc in MeOH	15 mL MeOH
Evaporation	Rotary evaporator to 2 mL followed by nitrogen stream	
Reconstitution	Reconstitution to 1 mL with MeOH	
Microfiltration (in case of wastewater)		
HPLC/DAD analysis	Modified method	Fist method

Fortification experiments with tap water and wastewater

The procedure for the extraction of PYR from tap water and wastewater with Oasis WCX (6 mL, 500 mg) is described in Table 3. Standard solution of PYR was added to 100 mL tap water samples with spiking level of 50 µg/L and to 100 mL wastewater samples with spiking levels of 50, 100 and 500 µg/L. Prior analysis with HPLC, this solution was filtered through a micro filter and transferred to an amber autosampler vial. Modified method was used for HPLC analysis of this experiment. The procedure to extract GAL, NEO, ESE and DON from tap water and wastewater with Oasis HLB (6 mL, 200 mg) is also described in Table 3. 200 mL tap water samples were spiked with a mixed standard solution at spiking level of 25 µg/L and 200 mL wastewater samples were spiked with a mixed standard solution at spiking level of 25, 50, 125 and 250 µg/L. In case of wastewater, the reconstituted solution was filtered through a micro filter then transferred to an amber autosampler vial. HPLC analysis for this experiment was performed with first method.

Calibration curves

Calibration curves for the determination of recovery rates were obtained by measuring calibration standards in five different concentrations 5.00, 10.0, 25.0, 50.0 and 100 ng/µL. Calibration standard solutions were prepared by dilution of a standard mixture of 100 ng/µL in MeOH. A linear regression was calculated using the peak area of the target substances. The linearity of the calibration curves was validated by the correlation coefficient of the linear regression (R^2).

Accuracy and precision

For the determination of the accuracy and the precision of the method, fortification experiments were conducted for wastewater and tap water in 4 replicates. The accuracy is defined as the mean value of the recovery rates (R = mean of measured concentrations/spiked concentration)·100% (Maláková et al., 2007; Furukori et al., 2002). The precision is expressed as relative standard deviation (RSD = (SD/mean)·100%) of the recovery rate of each substance. According to the criteria of DG Sanco [16], acceptable mean recovery rates are in the range of 70 – 120% with a RSD ≤ 20%.

Instrumental detection and quantification limits

In order to calculate the instrumental detection and quantification limits (IDL and IQL) a series of mixed standard solutions with equidistance concentrations from 0.25 to 3.00 ng/µL were prepared by dilution of a 50 ng/µL mixed standard solution. The IDLs are defined as the absolute concentrations of standard solution giving a signal-to-noise ratio (S/N) of 3. The IQLs are defined as the sample concentration of the standard solution giving a S/N of 10. The calculation was done by comparing the signal height of the baseline with the peak height of the target substances.

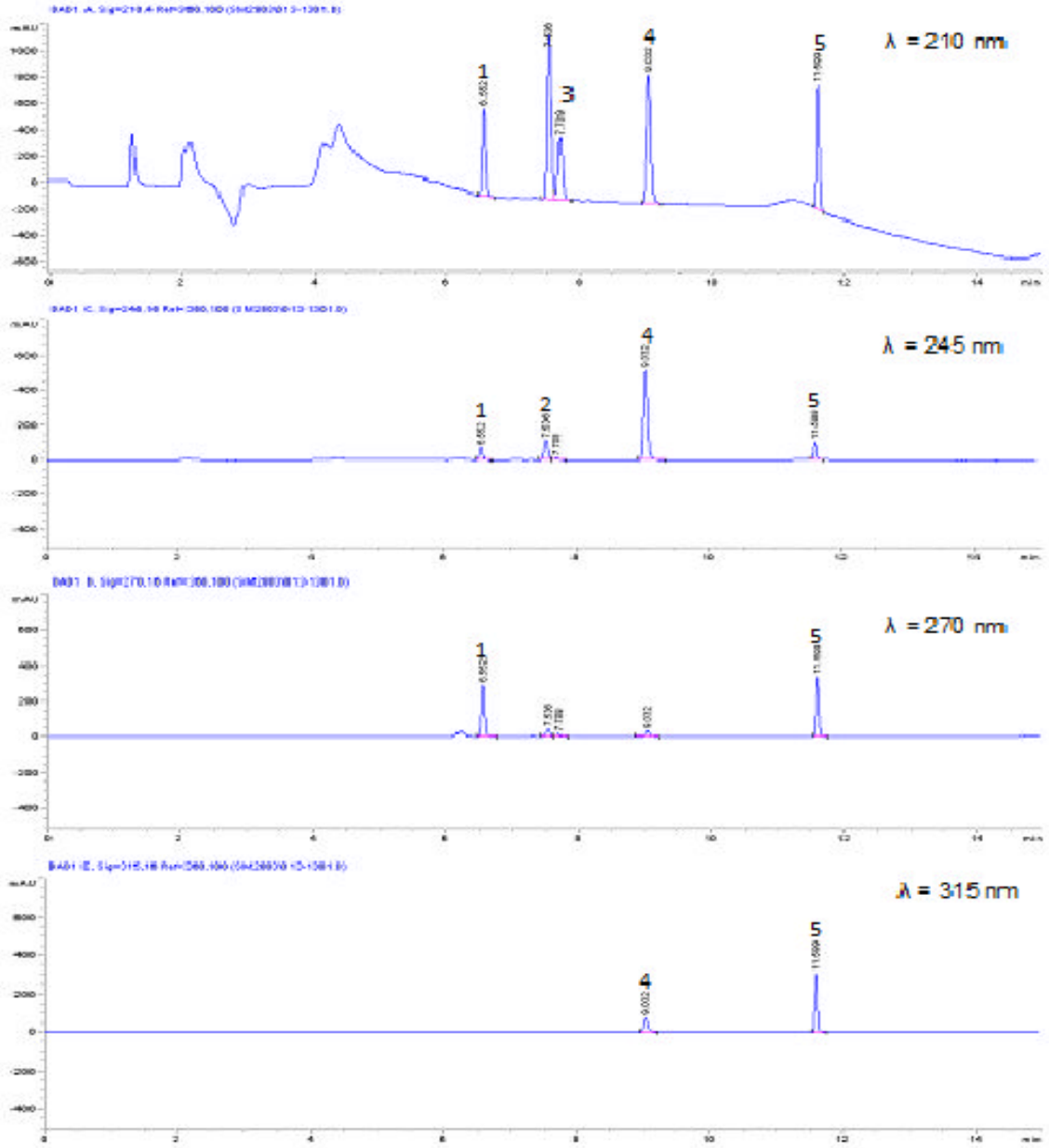


Fig 1. Chromatogram (λ=210, 245, 270 and 315 nm) of standard mixture 100 ng/µL using Symmetry Shield, 150 mm x 4.6 mm, 3.5 µm, gradient E with mobile phase A: H_2O/ACN (99:1, v/v) containing 0.1% HAc and 10 mM sodium 1-hexanesulfonate (pH = 3.1 ± 0.1) and mobile phase B: CAN. 1: PYR, 2: GAL, 3: NEO, 4: ESE, 5: DON

Method quantification limit

The method quantification limits (MQLs) were determined by fortification experiments with tap water and wastewater samples with different spiking level to the minimum level at which the analyte could be reliably quantified. 200 mL tap water samples were spiked with a mixed standard solution at spiking level of 25 µg/L and 200 mL wastewater samples were spiked with a mixed standard solution at spiking level of 25, 50, 125 and 250 µg/L. The lowest spiking concentration, at which acceptable recovery rates in the range of 70-120% with RSD ≤ 20% [17] were reached, was defined as method quantification limit.

RESULT AND DISCUSSION

Development of HPLC/DAD Method

The best separation with sharp symmetrical peaks was obtained using Symmetry Shield RP-18 (4.6 x 150 mm, particle size 3.5 µm) with mobile phase H_2O/ACN (95:5, v/v) containing 10 mM sodium hexane

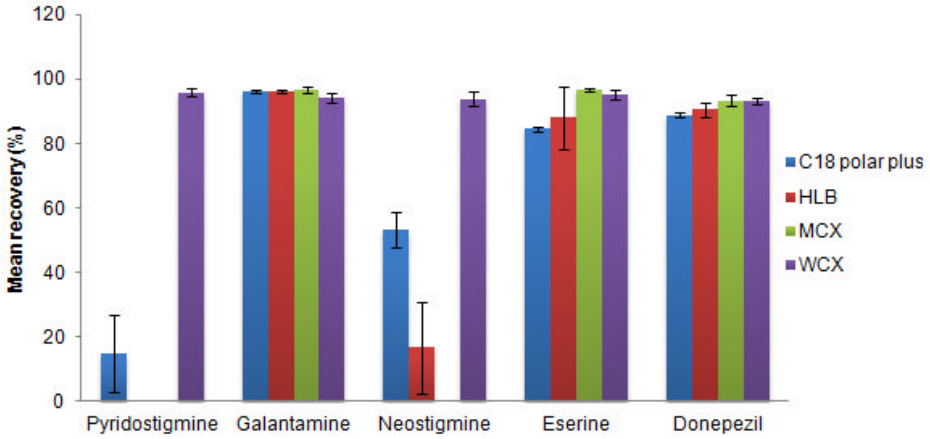


Fig 2. Comparison of recovery rates with C18 polar plus (6 mL, 500 mg), Oasis HLB (3 mL, 60 mg), Oasis MCX (3 mL, 60 mg) and Oasis WCX (6 mL, 150 mg) cartridges in 100 mL pure water spiked with 500 µL of 100 ng/µL mixed standard solution (c=500 µg/L). RSD are given as error bars

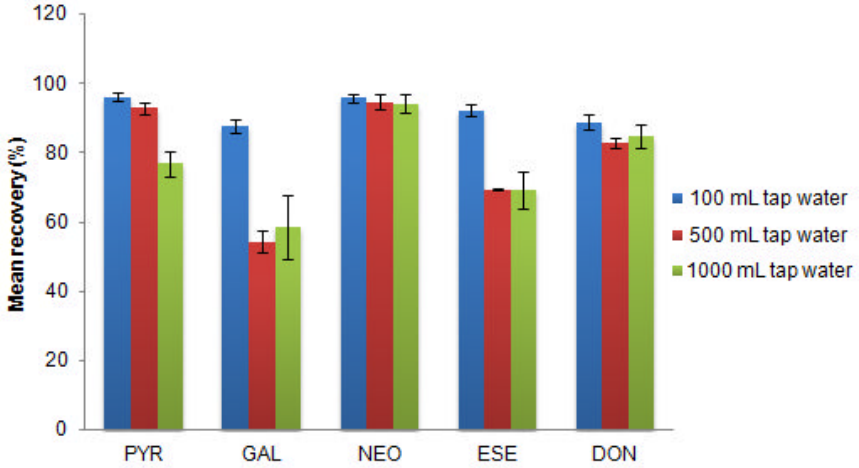


Fig 3. Mean recovery rates of target compounds from increasing volumes of 100 mL (c=500 µg/L) (n=4), 500 mL (c=100 µg/L) (n=2) and 1000 mL (c=50 µg/L) (n=2) spiked tap water samples with WCX cartridge (6 mL, 500 mg). RSD are given as error bars

sulfonate with 0.1% HAc and mobile phase B: ACN. Gradient system started 0% of B, increase to 100% B in 20 min and hold for 10 min decrease back to 0% B in 55 min. The pH of mobile phase A was acidified to 3.00 ± 0.1 by adding 0.1% HAc with the aim to protonate all the analytes. The target compounds, which have pKa of ranged from 6.1 to 12.2, should be in the cation form below pH 4 (2 pH rule). Ion pair reagents, sodium 1-hexanesulfonate were used to increase the retention of the analytes. All target substances were detected as single sharp peaks (width 0.057 to 0.071 min) (Fig. 1). Retention times of PYR, GAL, NEO, ESE and DON were 6.734, 7.633, 7.754, 8.498 and 10.374 min, respectively.

Comparison of Four Different SPE Cartridges

Four different SPE cartridges C18 polar plus, Oasis HLB (both reversed phase SPE), Oasis MCX and Oasis WCX (both ion exchange SPE) were used for the fortification experiments in four replicates. The Oasis MCX, Oasis HLB and C18 polar plus sorbents show similar tendencies. High recovery rates above 80% were only obtained for GAL, ESE and DON. But these cartridges were not efficient to recover PYR and NEO. Only the Oasis WCX (6 mL, 150 mg) sorbent showed high recovery rates for all target compounds in the range of 93.4–96.1% with low relative standard deviations in the range of 0.8 to 2.1 (Fig. 2).

In case of Oasis HLB, PYR, which is quaternary amine, was not detected or the recovery rates were only 10.4% at pH 5.5. This behavior may be attributable to the strong polarity of PYR (log K_{ow} 0.9). In case of Oasis WCX, the pH of the sample was not adjusted (pH around 8.0) therefore the target compounds were in the equilibrium between ionic and molecular form. Thus the interaction of the target

Table 4. Mean recovery and RSD (n=4) of GAL, NEO, ESE and DON in fortification experiment with 200 mL of tap water and wastewater using Oasis HLB cartridge (6 mL, 200 mg) spiked with mixed standard solution at spiking levels of 5, 10, 25 and 50 mg/L

Target compounds	Tap water		Wastewater							
	25 µg/L		250 µg/L		125 µg/L		50 µg/L		25 µg/L	
	Rec (%)	RSD	Rec (%)	RSD	Rec (%)	RSD	Rec (%)	RSD	Rec (%)	RSD
GAL (210 nm)	95.6	8.5	103.1	1.1	105.8	2.9	97.1	1.4	141	14.4
NEO (210 nm)	101.5	8.4	93.3	1.5	97.6	2.5	98.6	12.1	134.6	3.1
ESE (245 nm)	88.9	9.2	96.2	1.3	98.4	3.4	96.9	9.2	83.3	21.8
DON (315 nm)	95.8	7.7	98.4	1.0	99.2	2.0	98.7	4.5	82.2	5.3

Table 5. Mean recovery and RSD (n=4) of PYR in fortification experiment with 100 mL of tap water and wastewater using Oasis WCX cartridge (6 mL, 500 mg) spiked with standard PYR at spiking level of 5, 10 and 50 mg/L

Target compounds	Tap water		Wastewater					
	50 µg/L		500 µg/L		100 µg/L		50 µg/L	
	Rec (%)	RSD	Rec (%)	RSD	Rec (%)	RSD	Rec (%)	RSD
PYR (210 nm)	103.3	1.0	96.8	2.0	95.5	7.2	89.8	15.5
PYR (270 nm)	105.7	2.0	102.6	4.0	105.2	2.6	87.6	22.4

Table 6. Calibration equation and linearity for the HPLC/DAD method

Target compounds	R^2	Equation
PYR	0.999	y = 25.46x + 35.79
GAL	0.998	y = 48.36x + 62.70
NEO	0.999	y = 21.24x + 26.67
ESE	0.998	y = 42.75x + 60.92
DON	0.998	y = 29.02x + 40.89

compound with the SPE sorbent based on combination between ionic interaction and van der waals interaction.

Determination of the Capacity of WCX Cartridges

The effect of varying the volumes of tap water (100, 500 and 1000 mL) in the loading step were investigated in order to predict the extraction capacity of the Oasis WCX cartridge (6 mL, 500 mg). The recovery rates of NEO and DON were nearly unaffected by the volume of the tap water samples (Fig. 3). However, the recovery rates of PYR were slightly reduced with 500 mL tap water to 93% and further in 1000 mL tap water to 77%. Moreover, the recovery rates of GAL and ESE were drastically reduced with increasing volumes of tap water samples from 88% to 54% and from 92% to 69%.

The reductions of recovery rate of GAL and ESE were not observed when volumes of 500 mL sample were extracted by Oasis HLB (6 mL, 200 mg). Using this larger cartridge size, high recovery rates that ranged from 88.8 to 92.1% except for PYR were obtained. With regard to results with Oasis WCX and Oasis HLB cartridges, the extraction procedure was divided into 2 parts: For PYR the extraction with Oasis WCX (6 mL, 500 mg) was carried out and for GAL, NEO, ESE and DON, Oasis HLB (6 mL, 200 mg) was used.

Fortification Experiments with Tap Water and Wastewater Samples (Accuracy, Precision and Method Quantification Limit)

The optimized SPE procedures and the HPLC/DAD method were applied on spiked tap water and wastewater samples in order to determine the accuracy, the precision, and the quantification limit of the whole method. The fortification experiments for GAL, NEO, ESE and DON were conducted with Oasis HLB (6 mL, 200 mg) cartridges at spiking levels 25 µg/L for tap water and at four spiking levels of 25, 50, 125 and 250 µg/L for wastewater (Table 4). The recovery rates of the target substances from tap water were in the range of 88.8 to 101.5% while the RSD was in the range of 1.0 to 9.2. According to the criteria of DG Sanco [16], the results were in the acceptable range of 70-120% with RSD ≤ 20%. The recovery rates of the target substances in wastewater samples for spiking levels of 50, 125 and 250 µg/L were also in the acceptable range of DG Sanco [16]. They ranged between 93.3 and 105.8%, and the RSD values ranged between 1.0 and 12.1%. The RSD values increased in parallel to the decrease of the spiking level. At lowest spiking level of 25 µg/L, the recovery rates of GAL and NEO increased to 141 and 134% due to matrix interferences and thus were out of the acceptable range. While the recovery rates of ESE and DON decreased to 82.2% and 83.3%, which was still in the acceptable range. But the RSD of ESE was with 21.8% out of the acceptable range whereas the RSD of DON was with 5.3% still in the acceptable range.

The fortification experiments for analysis of PYR with Oasis WCX cartridges were conducted at spiking levels of 50 µg/L for tap water and 50, 100, 500 µg/L for wastewater samples. The recovery rates of PYR were

Table 7. IDL and IQL values of AChE inhibitors at 210, 245 and 270 nm

Wavelength	210 nm		245 nm		270 nm	
Target compounds	IDL (ng/µL)	IQL (ng/µL)	IDL (ng/µL)	IQL (ng/µL)	IDL (ng/µL)	IQL (ng/µL)
PYR	1.25	3.00	-	-	1.00	2.75
GAL	0.50	1.50	-	-	-	-
NEO	1.00	3.00	-	-	-	-
ESE	0.50	1.75	0.75	2.00	-	-
DON	1.00	2.50	-	-	0.75	2.00

measured at 210 and 270 nm because at low spiking level the interference from the matrix disturbs the PYR peak and may cause false positive detection of the target compounds. In tap water samples measured at wavelength of 210 and 270 nm, the recovery rates were in the range of 103.3 and 105.7%, respectively and the RSD was in the range of 1.0 and 2.0% (Table 5). The recovery rates of PYR from wastewater samples were conducted at the spiking levels of 100 and 500 µg/L. They ranged from 95.5 to 105.2% and RSD ranged from 2.0 to 7.2% indicating a good precision. However, at the spiking level of 50 µg/L, the recovery rate of PYR was decreased obviously to 87.8% (270 nm) and with an RSD above 20%.

In the frame of this work, the method quantification limits of the target substances were given as the lowest concentration, for which acceptable recovery rates and RSD values were obtained according to the criteria of DG Sanco [16]. In case of tap water, the lowest concentrations of 25 µg/L were still in the acceptable range for all target substances. Therefore, the MQL is at least 25 µg/L. Lower MQL might be reached for GAL, NEO, ESE and DON if higher volumes of tap water up to 1000 mL would be used for SPE. In case of wastewater for GAL, NEO, ESE and PYR (at 210 nm) the MQL was reached at a concentration of 50 µg/L whereas DON could be still quantified with acceptable results at concentration of 25 µg/L.

Calibration Curves

For the calibration of the target substances, five concentrations of 5, 10, 25, 50 and 100 ng/µL were measured with HPLC/DAD. Calibration data for the five substances are shown in Table 6. The correlation coefficients of the linear regression (R^2) were higher than 0.998 for all substances indicating a good linearity of the calibration functions in the measured concentration range. The slopes of the equations indicate the absorption intensity of the different target substances. The highest slopes of 48.36 and 42.75 were calculated for GAL and ESE. The linear regressions of the other substances had about 50% lower slopes.

Instrumental Detection and Quantification Limits

The instrumental detection and quantification limits (IDL and IQL) of all substances were in the range of 0.50 to 1.25 ng/µL and 1.5 to 3.0 ng/µL, respectively (Table 7). The sensitivity HPLC/DAD was different for the AChE inhibitors. HPLC/DAD has the highest sensitivity for GAL and ESE with IDL of 0.5 ng/µL and less sensitive for PYR with IDL 1.25 ng/µL.

CONCLUSION

HPLC/DAD method was developed for the analysis of the 5 AChE inhibitors, PYR, GAL, NEO, ESE and DON in water samples. Ion pair chromatography using sodium 1-hexanesulfonate was used for detection and separation of the target compounds. Fortification experiments with the Oasis WCX cartridge (6 mL, 500 mg) and Oasis HLB (6 mL, 200 mg) was selected for extraction and clean up the water samples. The results of SPE and HPLC/DAD method consistently demonstrated that the accuracy and precision meet the acceptance criteria of recovery rate of 70-120% and RSD ≤ 20%. The HPLC/DAD method was linear between concentrations of 5 to 100 ng/µL. The IDL and IQL ranged from 0.50 to 1.25 ng/µL and 1.5 to 3.0 ng/µL, respectively. The MQL for GAL, NEO, ESE and PYR (at 210 nm) in wastewater was reached for a concentration of 50 µg/L and DON at concentration of 25 µg/L. The MQL of 25 µg/L was acceptable for all target compounds in tap water. The developed SPE and HPLC/DAD method is applicable for quantification of the five target compounds in water samples in a concentration range > 25 µg/L and assumable lower for DON. The SPE followed with LC/MS/MS method would improve the sensitivity of the analytical method and therefore it may become the method of choice to quantify pharmaceuticals in water samples.

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