European Journal of Medicinal Chemistry 46 (2011) 3250–3257

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis, biological activity and HPLC validation of 1,2,3,4-tetrahydroacridine derivatives as acetylcholinesterase inhibitors

Paweł Szymanski [a,*], Adam Karpiński [b], Elzbieta Mikiciuk-Olasik [a]

[a] Medical University, Department of Pharmaceutical Chemistry and Drug Analyses, ul. Muszyńskiego 1, 90-151 Lodz, Poland
[b] Gerard Laboratories a Mylan Inc., Quality Control Department, Baldoye Industrial Estate, Unit 80, Dublin 13, Co. Dublin, Ireland

ARTICLE INFO

Article history:
Received 31 January 2011
Received in revised form
7 April 2011
Accepted 12 April 2011
Available online 22 April 2011

Keywords:
Tacrine
Alzheimer's disease (AD)
Acetylcholinesterase
Butyrylcholinesterase
HPLC validation
4-Fluoroacetic acid

ABSTRACT

The synthesis and biochemical evaluation of new hybrids of tacrine (THA) and 4-fluorobenzoic acid (4-FBA) possessing activity towards acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) inhibition are presented. The compounds of interest were obtained from the reaction of activated 4-FBA and diamino derivatives of 1,2,3,4-tetrahydroacridine. The compounds C6-2KW/HCl, C6-4KW/HCl and C6-3KW/HCl have four-fold higher antiacetylcholinesterase activity than THA. All of the acquired compounds present higher selectivity towards AChE than THA and lower selectivity towards BuChE. In addition, a rapid, selective and stability-indicating HPLC method was developed and validated for the determination of C6-2KW/HCl, C6-3KW/HCl and C6-4KW/HCl. THA and 4-FBA were found to be the main impurities. Chromatographic separation was achieved isocratically on a Waters Symmetry C18 150 × 3.9 mm, 4 μm column with a mobile phase of acetonitrile/buffer (17 mM sodium dodecyl sulphate and 8.3 mM sodium dihydrogen phosphate, 50:50 v/v) (overall pH 4). A 1.5 ml/min flow rate and a 247 nm wavelength were chosen for this method. C6-2KW/HCl, C6-3KW/HCl and C6-4KW/HCl were subjected to acidic and basic hydrolysis, chemical oxidation, thermal exposition at 60 °C and intense UV light. The limits of detection (LOD) and quantification (LOQ) were less than 2 μg/ml and 6 μg/ml for C6-2KW/HCl, C6-3KW/HCl and C6-4KW/HCl, 0.04 μg/ml and 0.12 μg/ml for THA, 0.42 μg/ml and 1.41 μg/ml for 4-FBA, respectively.

1. Introduction

Alzheimer's disease (AD) is a degenerative disease of the central nervous system, characterised by a noticeable cognitive decline and defined by a loss of memory and learning ability and a reduced ability to perform the basic activities of daily living. The diagnosis of AD is very difficult and still under development [1,2] or in clinical studies [3,4]. The testing of intellectual functions to determine the degree of cognitive status during medical examination is useful as a supplementary method of diagnosing dementia. Incorporating nuclear medicine into the diagnosis of dementia may not only lead to improvements in the quality of medical examinations but could also help to improve our understanding of the roots and development of the disease. To investigate drug delivery and define the mechanism of action, some atoms in a pharmaceutical ingredient can be replaced by their radioisotopes (e.g., ^{18}F) and then investigated in the body [5].

One of the most popular therapeutic strategies in AD is the control of cholinergic neurotransmission by the slow decline of neuronal degeneration or increasing cholinergic transmission [6]. The reduction in the activity of cholinergic neurons have used acetylcholinesterase inhibitors decreases the rate at which acetylcholine (ACh) is broken down, thereby increasing the concentration of ACh in the brain. Cholinesterase inhibitors have been approved as an efficacious treatment to reduce the symptoms of early-medium stages of AD. Well known commercial ACh inhibitors like tacrine, donepezil, rivastigmine and galantamine provide some evidence for their applicability in the advanced stages [7–9].

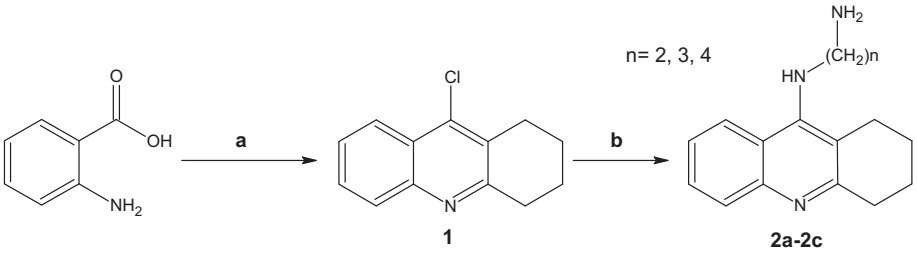
Current research is going towards new types of compounds, modelled by molecular modelling to estimate structure and inhibition activity towards AChE/BuChE [10–12].

Gonzales-Munoz and co-workers [13] presented a series of N-Acylaminophenothiazines as a potential BuChE inhibitors. Similar activity was observed by Tasso [14] with quinolizidinyl derivatives of bi- and tricyclic structures.

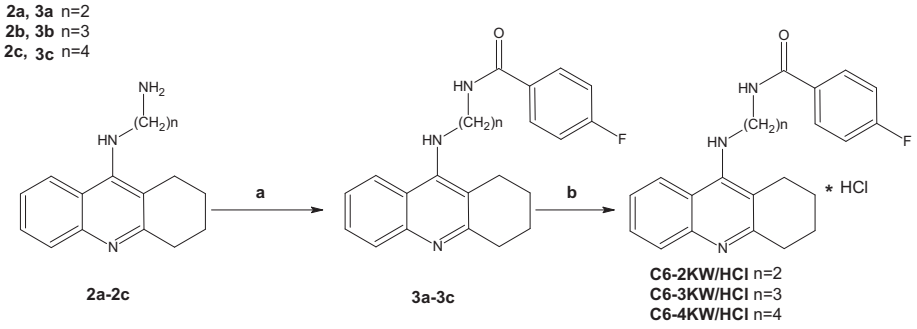
Gholivand [15] provide interesting studies about new phosphorus(V) hydrazides. Synthesised compounds have not only some antibacterial activity but are able to slow down AChE and BuChE (mainly) activity measured by Ellman's method [16].

* Corresponding author. Tel./fax: +48 42 677 92 50.
E-mail addresses: pawel.szymanski@umed.lodz.pl (P. Szymanski), adas_chem@yahoo.pl (A. Karpiński).

0223-5234/$ – see front matter © 2011 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.ejmech.2011.04.038



Scheme 1. Synthesis of compounds **1**, **2a**–**2c**. Reagents: (a) cyclohexanone, $POCl_3$, reflux; (b) 1,ω-diamine, phenol, NaI, reflux.



Scheme 2. Synthesis of final compounds. Reagents: (a) 4-fluorobenzoic acid, CDMT, methylmorpholine, THF; (b) HCl/ether.

However, there is intensive research towards a new type of drug based on tacrine, which was registered by the American FDA as the first drug in AD treatment. The therapeutic effects of tacrine for the treatment of cognitive defects in Alzheimer's disease (AD) were reported in 1986 [17]. Newly designed drugs must eliminate tacrine side effects like hepatotoxicity and cardiovascular system impairment and also increase oral bioavailability.

The THA derivatives as AChE/BuChE inhibitors were often developed. In 2004 Frideling [18] and co-workers synthesised chiral THA derivatives from 3-methylcyclohexananone, menthone, pulegone, carvone and dihydrocarvone. Pisoni [19] proposed enentioselective synthesis of THA derivatives where inhibition activity has been checked (IC50 = 0.06–7.22 μM).

The aim of this work was the synthesis of compounds tested by molecular-modelling techniques, the calculation of biological activity and the development and validation of a rapid reversed-phase HPLC-UV method that will be useful for further early-stage pharmaceutical testing.

Previously developed HPLC methods for tacrine, derivatives and its metabolites have used fluorescence detection with a polar stationary phases at a LOD of 0.25 ng/ml to 5.95 ng/ml [20–22].

2. Experimental

2.1. Materials

1,2,3,4-Tetrahydroacridin-9-amine trihydrate (Tacrine, 99%) and 4-fluorobenzoic acid (>99%) were received from Sigma–Aldrich GmbH (Steinheim, Germany). The reactions were monitored by TLC using 25 DC-Alufolien Kieselgel 60F_{254} (Merck KGaA, Damstad, Germany) and a UV lamp (254 nm). The melting points were measured on an electrothermal apparatus with open capillaries. Anhydrous sodium sulphate (Sigma–Aldrich GmbH, Steinheim, Germany) was used to dry organic solutions during the work-up, and the removal of solvents was carried out under vacuum with a rotary evaporator. Column chromatography was performed using silica-gel 60 (230–400 mesh, Merck KGaA, Damstad, Germany).

DTNB, both enzymes (C2629 and C4290) and acetylthiocholine iodide were purchased from Sigma–Aldrich GmbH, Steinheim, Germany for biological-activity calculation.

Samples of C6-2KW/HCl (4-fluoro-*N*-[2-(1,2,3,4-tetrahydroacridin-9-ylamino)ethyl]-benzamide hydrochloride), C6-3KW/HCl (4-fluoro-*N*-[3-(1,2,3,4-tetrahydroacridin-9-ylamino)propyl]-benzamide hydrochloride), and C6-4KW/HCl (4-fluoro-*N*-[4-(1,2,3,4-tetrahydroacridin-9-ylamino)butyl]-benzamide hydrochloride) were synthesised in-house (see Supplementary data, [23]). In addition compounds were purified for validation purposes using micro scale Dry Column Chromatography (DCC) [25].

HPLC-grade water and sodium dihydrogen phosphate (Sigma–Aldrich GmbH, Steinheim, Germany), HPLC-grade acetonitrile (Merck KGaA, Damstad, Germany) and sodium dodecyl sulphate (Romil LTD, Cambridge, UK) were used.

2.2. Synthesis

The first step of the synthesis (Scheme 1) involves the reaction between anthranilic acid and cyclohexanone in freshly prepared

Table 1
Statistical parameters value of K_m and V_{max} for AChE and BChE.

Parameters	AChE	BChE
K_m	0.092683 μM	0.100176 μM
V_{max}	2.29645 μM/min/ml	2.526429 μM/min/ml
r^2	0.9954	0.9858
Standard error	0.098	0.021

Table 2
IC_{50} values for activities on acetylcholinesterase and butyrylcholinesterase.

Compounds	AChE inhibition IC_{50}, μM	BChE inhibition IC_{50}, μM	Selectivity for AChE[a]	Selectivity for BChE[b]
C6-2KW/HCl	8.15×10^{-4}	4.49×10^{-5}	0.047	21.27
C6-3KW/HCl	8.09×10^{-4}	2.12×10^{-5}	0.033	30.72
C6-4KW/HCl	7.84×10^{-4}	3.54×10^{-5}	0.047	21.17
THA	3.16×10^{-3}	2.67×10^{-5}	0.008	118.63

[a] Selectivity for AChE is defined as IC_{50}(BChE)/IC_{50}(AChE).
[b] Selectivity for BChE is defined as IC_{50}(AChE)/IC_{50}(BChE).

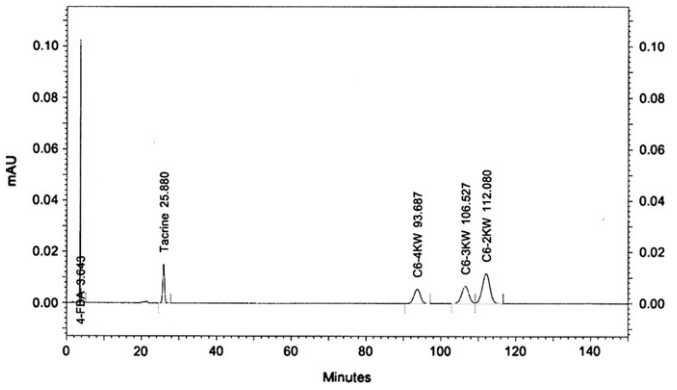


Fig. 1. Separation of tested compounds: Waters Symmetry C18, 250 mm × 4.6 mm, 4 μm, 30 °C, mobile phase: 62.5–37.5% (buffer*-ACN, v/v, *17 mM SDS and 8.3 mM NaH_2PO_4) (overall pH 4).

$POCl_3$. A similar reaction was reported by M.K. Hu and C.F. Lu [26]. The efficiency of this reaction was about 54%. The obtained compound, **1**, was then combined with two equivalents of the appropriate 1,ω-diamine. This reaction was accompanied by the addition of sodium iodide (catalyst) in the presence of phenol at 180 °C. The yields of this reaction were: 91% (**2a**), 88% (**2b**) and 72% (**3c**). The generated compounds were subsequently used as substrates for further synthesis (Scheme 2). The synthesis of compounds **3a**–**3c** have involved the activation of 4-fluorobenzoic acid by stoichiometric amounts of 2-chloro-4,6-dimethyl-1,3,5-triazine (CMDT) and N-methylmorpholine at low temperature [27]. We report that the results of this reaction stage are significantly better when N-methylmorpholine is added dropwise into the solution in an adequate solvent, such as dichloromethane, acetonitrile, dioxane or tetrahydrofuran, at −5 °C. The progress of this reaction was verified by TLC. After approximately 2 h, a solution of compounds **2a**–**2c** in the proper solvent was added to the activated 4-fluorobenzoic acid. The final step of the synthesis was conducted in the presence of hydrochloric acid in ether to produce the hydrochloride form of the products (C6-2KW/HCl (**3a**), C6-3KW/HCl (**3b**) and C6-4KW/HCl (**3c**)).

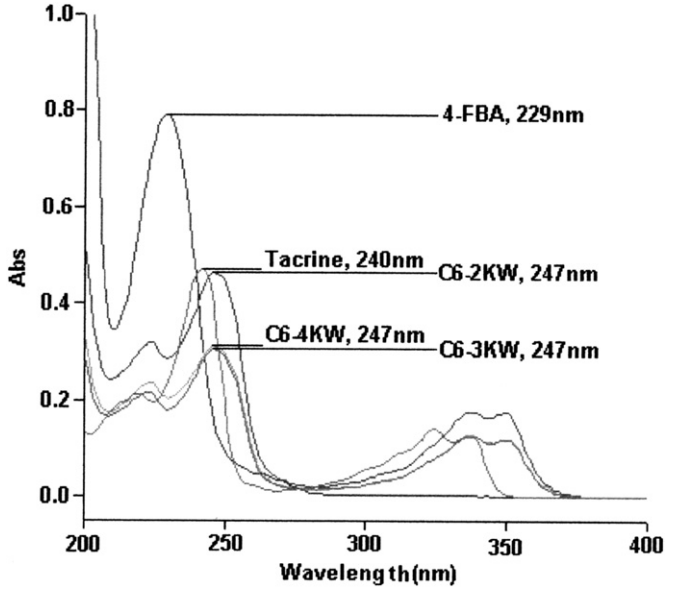


Fig. 2. UV-absorption spectra of 4-FBA, THA and derivatives from 200 nm to 400 nm. Concentration: C6-2KW/HCl, C6-3KW/HCl and C6-4KW/HCl at 5 μg/ml, 4-FBA at 10 μg/ml and THA at 2.5 μg/ml.

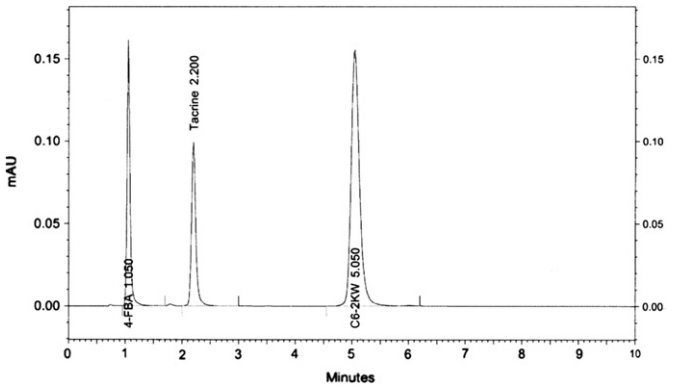


Fig. 3. A typical chromatogram of the separation of 4-FBA, tacrine and C6-2KW/HCl.

2.3. Biological activity

Ellman's spectrophotometrical method [16] was used with some modifications to determine the activity of the newly synthesised acetylcholinesterase inhibitors. Measurements were conducted at 37 °C in phosphate-buffered solution (0.1 M, pH 8.0). The reaction mixture in a final volume of 3 ml contained the AChE enzyme (5 units/ml) and 5,5′-dithiobisnitrobenzoic acid (DTNB, 0.05 ml, 0.5 M), which is a chromogen and an adequate inhibitor. Acetylthiocholine iodide was used as a substrate. The inhibition curves for every derivative were made with seven concentrations of acetylthiocholine iodide. The activity of the acquired derivatives was marked by measuring the absorbance at 412 nm. The measurement was taken after 1 min of incubation of the reaction mixture. Each measurement was made in triplicate. To acquire all of the AChE, activity tests without inhibitors were always done.

The activity towards the inhibition of butyrylcholinesterase (BuChE) was determined in the same way as that for AChE. In these experiments, BuChE was used in the same concentration as AChE (5 units/ml).

Non-linear and linear regressions were used to estimate the drug concentration inducing 50% inhibition of the AChE or BuChE activity.

2.4. Apparatus

IR spectra were recorded in KBr using a Mattson Infinity Series FT-IR spectrophotometer. ^{1}H NMR spectra were recorded with a Varian Mercury 300 MHz spectrometer using tetramethylsilane as an internal standard. Mass spectra and elemental analyses were

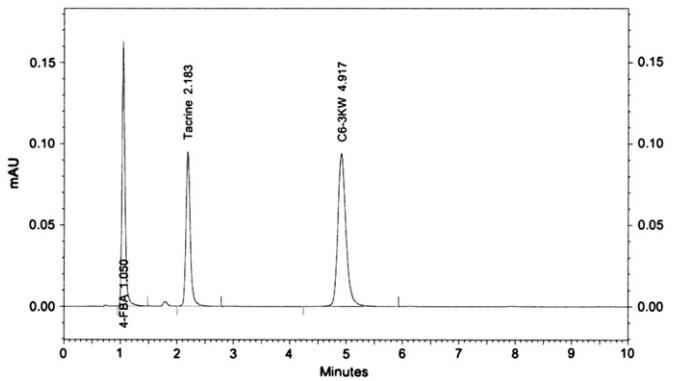


Fig. 4. A typical chromatogram of the separation of 4-FBA, tacrine and C6-3KW/HCl.

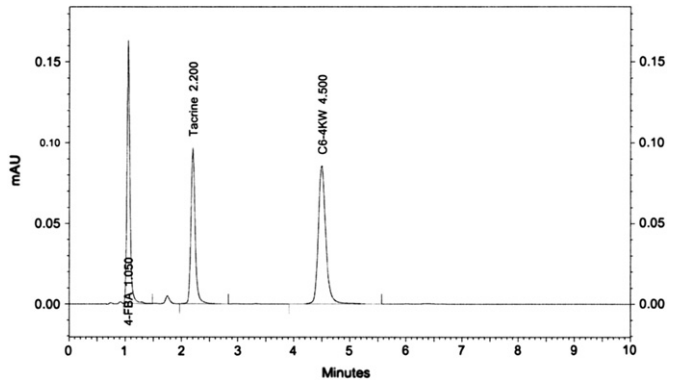


Fig. 5. A typical chromatogram of the separation of 4-FBA, tacrine and C6-4KW/HCl.

performed by the Centre of Molecular and Macromolecular Studies in Lodz (Polish Academy of Sciences).

The HPLC instrument consisted of a Waters 2695 Alliance separation module with a Waters 2487 VWD (variable-wavelength detector) and a Waters 2996 PDA detector (photodiode-array detector) (Waters Corp., Milford, USA). However, an Agilent 1200 series with a VWD detector (Agilent Technologies, Waldbronn, Germany) was used throughout the validation process. The software used for data collection, integration and computation was Millennium 32 (Waters) and Chromatography Data System (CDS) Software (Agilent). A reversed phase Symmetry C18 column (150 mm × 3.9 mm i.d.; particle size 4 μm) (Waters, USA) was used for the separation.

2.5. Chromatographic conditions

The mobile phase consisted of acetonitrile and buffer (50:50, v/v). The buffer consisted of 17 mM SDS and 8.3 mM sodium dihydrogen phosphate. The overall pH was set to 4 ± 0.2 with phosphoric acid. Before injection into the system, mobile phase was filtered through a 0.45 μm filter and degassed under vacuum. The analysis was carried out under isocratic conditions using a flow rate of 1.5 ml/min at ambient temperature with an injection volume of 10 μl. Chromatograms were recorded at 247 nm. A mixture of the acetonitrile and water (50:50, v/v) was used as the diluent.

The PDA detector was used during the validation procedure.

2.6. Preparation of solutions

A stock solution of 0.5 mg/ml was prepared by dissolving the appropriate amount of the sample (C6-2KW/HCl, C6-3KW/HCl, or C6-4KW/HCl) in the diluent. A working solution of 0.05 mg/ml was prepared from the stock solution for the related-substance and assay determination. Stock solutions of THA at 0.1 mg/ml and 4-FBA at 1 mg/ml were also prepared in the diluent. Working impurity solutions were prepared by 1:10 dilution in diluent for the related-substance determination. Appropriate dilutions of stock solutions were used for linearity studies and assay purposes.

Table 4
Method precision summary (intra and inter-assay).

Compound	RSD (%) Method precision	Intermediate precision (%)
4-FBA	0.90	0.91
THA	0.48	0.51
C6-2KW/HCl	0.56	0.65
C6-3KW/HCl	0.41	0.72
C6-4KW/HCl	0.62	0.39

2.7. Method validation

The analytical-method validation was carried out as per the ICH Q2(R1) method-validation guidelines [24]. The following validation parameters were addressed: selectivity, precision, linearity, accuracy, limit of detection, limit of quantification, robustness and the stability of the tested compounds in diluent.

3. Results and discussion

3.1. Biochemical studies

The spectrophotometrical method pioneered by Ellman was used to determine the inhibitory activity of the synthesised compounds towards acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) [16]. The inhibition type was justified by the linear Lineweaver–Burk equation. The constant parameters K_m and V_{max} were evaluated by the linear regression of the reaction rate versus the substrate concentration. A non-linear regression was used to estimate the apparent K_i constants. In Table 1, the statistical parameters and values of K_m and V_{max} are presented for both AChE and BuChE enzymes. Table 2 shows the acquired data of the inhibitory activities of C6-2KW/HCl, C6-3KW/HCl and C6-4KW/HCl towards AChE and BuChE. Newly synthesised constituents C6-2KW/HCl, C6-3KW/HCl and C6-4KW/HCl, in comparison with tacrine, possess 4-fold higher inhibitory activity towards AChE (3.88, 3.91, 4.03 respectively). For all of the obtained compounds, slightly higher IC_{50} values were recorded for BuChE than for tacrine. Our structural modifications focused on the amino group of tacrine: small aliphatic chain was inserted and coupling with 4-fluorobenzoic acid to assess the importance change in this part of molecules. Compounds C6-2KW/HCl, C6-3KW/HCl and C6-4KW/HCl showed a remarkable increase in activity, underlying the importance of the longer alifatic chain. This shows that it was good way to introduce this alteration.

Furthermore, we estimated the selectivity of the inhibitory effects of all received components and tacrine towards acetylcholinesterase (ratio of IC_{50} BuChE/AChE) and butyrylcholinesterase (ratio of IC_{50} AChE/BuChE). All synthesised compounds display higher selectivity towards AChE than tacrine (between 4.13 and 5.88-fold more selective). For the inhibition of BuChE, tacrine presented the highest selectivity than AChE. Generally it was shown that after coupling tacrine with fluorobenzoic acid, we obtained compounds more selective to BuChE. This is very important because it is know that in Alzheimer Disease we can observe higher

Table 3
System precision summary.

Compound	RSD(%) Day 1	RSD(%) Day 2	RSD(%) Day 10	RSD(%) Separate HPLC	Theoretical plates (average)	Asymmetry (average)
4-FBA	0.08	0.21	0.14	0.11	1482	1.3
THA	0.27	0.22	0.15	0.31	4217	1.3
C6-2KW/HCl	0.05	0.06	0.09	0.09	5994	1.3
C6-3KW/HCl	0.11	0.09	0.10	0.01	6030	1.2
C6-4KW/HCl	0.13	0.10	0.15	0.23	5810	1.2

Table 5
Linearity results.

Compound	Range μg/ml	R^2	Slope	CI of the slope	Intercept	LOD μg/ml	LOQ μg/ml
C6-2KW/HCl	5–200	0.99995	28380	277	−21414	1.96	5.94
C6-3KW/HCl	5–200	0.99999	17918	76	−8429	0.84	2.55
C6-4KW/HCl	5–200	0.99999	15296	11	−8026	0.81	2.45
THA	0.05–10	0.99999	44412	174	153	0.04	0.12
4-FBA	0.5–100	0.99999	3730	6	568	0.42	1.41

Table 6
Summary for recovery of tacrine derivatives in diluent.

	C6-2KW/HCl (%)	C6-3KW/HCl (%)	C6-4KW/HCl (%)
80%	101.46 ± 0.46	99.41 ± 0.95	99.15 ± 0.57
100%	100.39 ± 0.43	99.40 ± 0.61	99.03 ± 0.39
120%	100.82 ± 0.92	99.02 ± 0.93	98.71 ± 1.04

For each level samples were prepared in triplicate, and presented as average of content.

level of this enzyme in central nervous system. We can claim that this tag of these compounds could be strong advantage as a new potential AChEI's.

3.2. Validation and method development

During method development, a non-buffered mobile phase was investigated in gradient mode, but both the separation and elution time were unsatisfactory. Instead, a mobile phase consisting of a SDS-buffer and acetonitrile switch C18 column was chosen. This procedure improved the separation between base compounds via octadecyl chains and the alkyl "bridge" of tacrine derivatives. The working pH of the mobile phase was determined using the estimated pKas from Marvin Software (ChemAxon) for all tested compounds. The mobile phase was prepared at a pH in the range of 3–4.2 by the addition of sodium dihydrogen phosphate and adjusted to 4 ± 0.2 with phosphoric acid. The use of this mobile phase led to a successful separation. Due to economical and practical considerations, a linear method was developed that is able to separate impurities (synthesis substrates) from tacrine derivatives.

During the method-development process, a separation test of all tacrine derivatives performed, including the optimisation of the mobile-phase composition and temperature with a Waters Symmetry column (250 mm × 4.6 mm, C18, 4 μm). However, due to its lengthy run time, this method has little practical application (Fig. 1).

3.3. Determination of a suitable wavelength

Prior to the chromatographic-method development, the detection wavelength was determined by obtaining UV spectra of 4-FBA, tacrine and tested compounds solutions. Samples were dissolved in diluent and scanned by a UV spectrophotometer in the range of 200–400 nm. The UV spectra of five substances are illustrated on Fig. 2.

The maximum absorbance for the three derivative substances appears at 247 nm compared to the tacrine maximum at 240 nm. However, the response of tacrine is twice as high as that of the derivatives. The maximum absorbance of 4-FBA appears at 229 nm, and it produces a four-fold smaller absorbance at 247 nm. However, it was concluded that 247 nm is the most appropriate wavelength for analysing the tested compounds with suitable sensitivity despite the smaller absorbance for 4-FBA. The linearity shows that this method is linear in range from 0.5 to 100 μg/ml.

Table 8
Recovery of 4-FBA and tacrine in C6-3KW/HCl solution.

	Impurity at 20% Spiking		Impurity at 100% Spiking		Impurity at 200% Spiking	
Sample	4-FBA	THA	4-FBA	THA	4-FBA	THA
1	102.12	108.81	100.89	101.45	98.07	99.48
2	100.98	108.77	101.86	101.74	97.63	99.82
3	100.90	108.98	100.89	101.75	98.05	99.98
Mean	101.33	108.85	101.21	101.65	97.92	99.76
SD	0.68	0.11	0.56	0.17	0.25	0.26
R.S.D. (%)	0.67	0.10	0.55	0.16	0.25	0.26

3.4. Selectivity

The HPLC chromatograms recorded for the mixture of C6-2KW/HCl (C6-3KW/HCl or C6-4KW/HCl), 4-FBA and THA (Figs. 3–5) clearly show that the tacrine derivatives are fully separated from the related compounds. The resolution factor obtained for 4-FBA and THA was 10.4, and the resolution factors for THA and the derivatives were 15.1 for C6-2KW/HCl, 14.6 for C6-3KW/HCl and 12.8 for C6-4KW/HCl. In this study, a PDA detector was used to confirm that the tacrine-derivative peaks are free from other co-eluting substances. It was concluded that the developed method is selective to impurities, mobile phase and diluent.

3.5. Precision and repeatability

The RSD (%) for the system precision was lower than 1% for all tests, which complies with the proposed acceptance criteria (NMT

Table 7
Recovery of 4-FBA and tacrine in C6-2KW/HCl solution.

	Impurity at 20% Spiking		Impurity at 100% Spiking		Impurity at 200% Spiking	
Sample	4-FBA	THA	4-FBA	THA	4-FBA	THA
1	98.51	94.52	99.88	96.79	100.42	97.97
2	97.65	91.67	99.80	96.72	100.53	97.93
3	97.83	91.62	101.44	96.50	100.74	100.06
Mean	97.99	92.60	100.37	96.67	100.56	98.65
SD	0.45	1.66	0.92	0.15	0.16	1.22
R.S.D. (%)	0.46	1.80	0.92	0.16	0.16	1.24

Table 9
Recovery of 4-FBA and tacrine in C6-4KW/HCl solution.

	Impurity at 20% Spiking		Impurity at 100% Spiking		Impurity at 200% Spiking	
Sample	4-FBA	THA	4-FBA	THA	4-FBA	THA
1	104.30	103.79	103.24	102.70	98.93	102.24
2	103.00	101.85	102.78	102.19	99.50	103.34
3	104.82	103.23	102.48	101.50	99.61	103.33
Mean	104.04	102.96	102.83	102.13	99.34	102.97
SD	0.94	1.00	0.38	0.60	0.37	0.63
R.S.D. (%)	0.90	0.97	0.37	0.59	0.37	0.61

Table 10
Summary of forced degradation results for C6-2KW/HCl.

Stress conditions	Time	% Assay of active substance	Remarks
Acid hydrolysis (0.5 M HCl at RT)	48 h	101.1%	No degradation products formed (total unknown less than 0.1%)
Base hydrolysis (0.5 M NaOH at RT)	48 h	99.7%	No degradation products formed (total unknown less than 0.1%)
Oxidation (3% H_2O_2 at RT)	48 h	100.3%	No degradation products formed (total unknown less than 0.1%)
Thermal 60 °C	8 h	100.5%	No degradation products formed (total unknown less than 0.1%)
UV (254 nm)	8 h	97.8%	No degradation products formed

Table 11
Summary of forced degradation results for C6-3KW/HCl.

Stress conditions	Time	% Assay of active substance	Remarks
Acid hydrolysis (0.5 M HCl at RT)	48 h	99.4%	No degradation products formed (total unknown less than 0.1%)
Base hydrolysis (0.5 M NaOH at RT)	48 h	99.8%	No degradation products formed (total unknown less than 0.2%)
Oxidation (3% H_2O_2 at RT)	48 h	100.3%	Some unknown degradation products were formed (total unknown about 0.3%)
Thermal 6 0 °C	8 h	100.8%	No degradation products formed (total unknown less than 0.1%)
UV (254 nm)	8 h	100.5%	No degradation products formed (total unknown less than 0.1%)

Table 12
Summary of forced degradation results for C6-4KW/HCl.

Stress conditions	Time	% Assay of active substance	Remarks
Acid hydrolysis (0.5 M HCl at RT)	48 h	99.5%	Some unknown degradation products were formed (total unknown about 0.4%)
Base hydrolysis (0.5 M NaOH at RT)	48 h	98.1%	Some unknown degradation products were formed (total unknown about 0.3%)
Oxidation (3% H_2O_2 at RT)	48 h	94.7%	Some unknown degradation products were formed (total unknown about 0.5%)
Thermal 60 °C	8 h	99.1%	Some unknown degradation products were formed (total unknown about 0.3%)
UV (254 nm)	8 h	102.1%	No degradation products formed (total unknown less than 0.1%)

2%). The method and intermediate have a relative standard deviation (RSD) below 1%, which also complies with the proposed criteria.

3.6. Stability of solutions

The standard solutions of the active samples and the impurities and a standard solution prepared from old standard stock were run on Day 1, Day 2 and Day 10. The standard stock solution and the working-standard solution were determined to be stable for up to ten days. There was no response difference between Day 1 and Day 10, and no degradation products were detected.

3.7. Linearity, LOD and LOQ

The calibration curves for 4-FBA, THA, C6-2KW/HCl, C6-3KW/HCl and C6-4KW/HCl were found to be linear with correlation coefficients greater than 0.999. Table 3 lists the linearity parameters of the calibration curves for tacrine and tacrine derivatives. Each point in the regression is the mean of three experiments.

The linear equations given in the linearity results were used to calculate the limit of detection (LOD) and the limit of quantification (LOQ). The LOD and LOQ are also given in this table and were found to be lower than 2 mg/ml for LOD and less than 6 mg/ml for LOQ of the tested tacrine derivatives.

3.8. Accuracy (% recovery)

The accuracy was determined by the recovery of the tacrine derivatives prepared at 80%, 100% and 120% of the working standard in triplicate. The recovery was found to be within 98–102% for all levels, which meets the proposed criteria. The summary results are presented in Table 4.

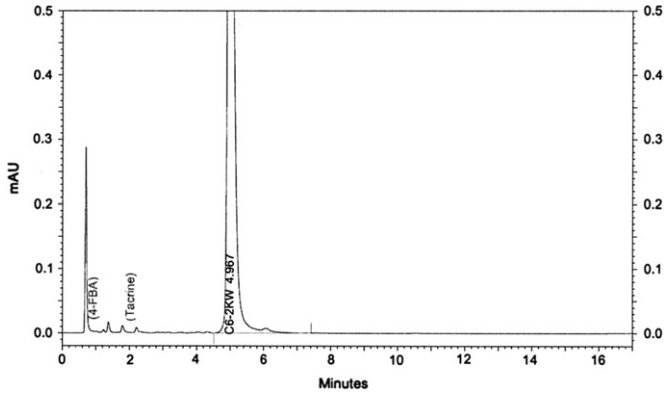


Fig. 6. Representative chromatography for C6-2KW/HCl at 0.5 mg/ml for the oxidation test with 3% H_2O_2. At 0.7 min, the blank peak is visible.

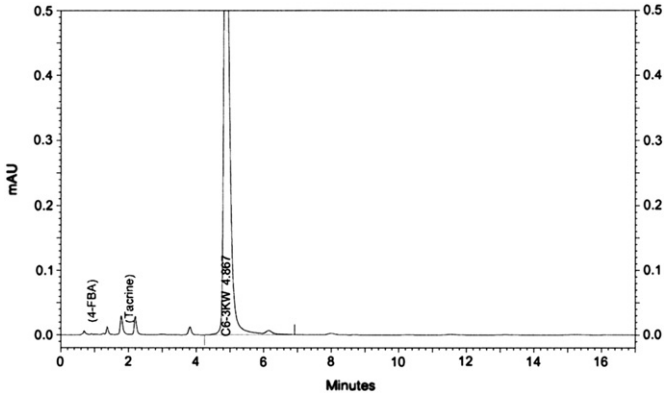


Fig. 7. Representative chromatography for C6-3KW/HCl at 0.5 mg/ml for the base test with NaOH.

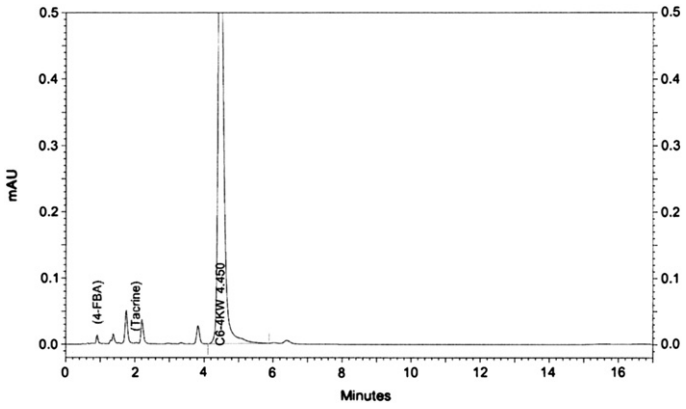


Fig. 8. Representative chromatography for C6-4KW/HCl at 0.5 mg/ml for the acid test with HCl.

To determine the accuracy of the related-substance method, sample solutions were spiked at three different concentrations: 20%, 100% and 200% of the working-standard concentration. Triplicate samples were prepared and analysed. The results shown in Tables 5–7, are expressed as percent recoveries of the particular sample components. All of the results were within specification: the recovery at the 20% level should be within 85–115% and that at the 100% and 200% levels should be within 90–110% (Tables 8–12).

3.9. Robustness

The method robustness checked after deliberate alterations of the mobile-phase composition, buffer composition, pH and flow shows that slight changes in the operational parameters do not lead to fundamental changes of the performance of the chromatographic system within the specified limits. The tailing factor for C6-2KW/HCl, C6-3KW/HCl, C6-4KW/HCl and Tacrine always ranged from 1 to 1.4, and the components were well separated under all of the changes performed. The percent recoveries of the tested compounds were good under most conditions and did not show a significant change when the critical parameters were modified. Considering the results of the modifications of the system-suitability parameters and the specificity of the method, it is concluded that the method conditions are robust.

3.10. Degradation study

For the acid hydrolysis, 0.2 ml of 1 M HCl per 5 ml of test solution was added and kept in ambient temperature for 48 h. After this time, the relevant volume of 1 M NaOH was added to neutralise the solution. The alkali hydrolysis used the same procedure with 1 M NaOH used for the degradation procedure for 48 h and 1 M HCl used for neutralisation. For the oxidation test, 0.2 ml of 5% H_2O_2 was added for each 5 ml of test solution, kept for 48 h and injected into the HPLC. For the UV-light degradation, a 0.5 mg/ml solution was prepared and exposed for 8 h to intense UV light (254 nm). For the thermal degradation, 0.5 mg/ml test solutions were kept for 8 h in a 60 °C water batch, cooled and injected into the HPLC system. In each case, a blank solution was prepared as per the degradation test.

All of the chromatograms of the degradation studies were analysed against chromatograms of fresh solutions and relevant blank solutions. All peaks from the fresh solutions were identified with the difference checked and calculated. In all base and H_2O_2 solutions, the blank peak at 0.7 min was visible. For the presented degradation studies, there is no evidence that conditions such as HCl, NaOH, H_2O_2, heat and UV lead to major degradation of tacrine derivatives. Tacrine and 4-FBA impurities were not observed during stress studies. Representative chromatograms of stress studies are presented on Figs. 6–8.

Photodiode-array investigations of the active peak after stress testing confirm the active peak to be pure, demonstrating the stability-indicating nature of the method over the full spectrum of stress testing. The total recovery of the active substance was between 94.7% and 102.1%.

4. Conclusions

Treatment of AD, the most common form of dementia among the elderly, still remains a challenge for scientists and doctors. In the recent decades, there has been an accelerating general effort to determine the risk factors and causes of AD development. Studies have also been conducted to find better ways of treating this illness and delaying its onset.

AChE inhibitors like tacrine, donepezil, galantamine and rivastigmine are established drugs in the treatment of AD. These medicines are commonly approved for mild- to moderate-AD treatment. However, the use of these drugs is frequently limited because of their adverse effects. Thus, there is still a need to search for new compounds with AChE inhibitory activity. A drug design allowed us to synthesise a new series of AChEIs as analogues of tacrine.

The acquired compounds C6-2KW/HCl, C6-3KW/HCl and C6-4KW/HCl are characterised by 4-fold higher activity towards AChE inhibition than the reference tacrine compound. Furthermore, all obtained components demonstrate better selectivity for AChE than does tacrine. The different structural modifications carried out on tacrine especially on amino group were able to improve the AChE inhibitory potency of the new derivatives. This finding is very significant, especially in the context of AD diagnosis, because this illness is associated with AChE deficits.

An isocratic RP-HPLC method has been developed and validated for the purity evaluation and the determination of tacrine derivatives. The developed method is selective, sensitive, precise and stability indicating. The method is also capable of detecting THA and 4-FBA at trace levels.

Synthesised in a simple and affordable way compounds shows a fairly good inhibitory activity. We have elaborated HPLC method for this compounds too, and that is way they could be considered as a new lead for further optimisation as a new potential AChEI.

Role of the funding source

Financial support from the Medical University (Grant No 503/3-015-01/503-01) is gratefully acknowledged.

Acknowledgements

Many thanks to Gerard Laboratories at Mylan Corp., for significant support of this research.

Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2011.04.038.

References

[1] N. O'Rourke, H. Tuokko, S. Hayden, B.L. Beattie, Early identification of dementia: predictive validity of clock test, Arch. Clin. Neuropsychol. 12/3 (1997) 257–267.
[2] J. De Lepeleire, J. Heyrman, F. Baro, F. Buntinx, A combination of tests for the diagnosis of dementia had a significant diagnostic value, J. Clin. Epidemiol. 58/3 (2005) 217–225.
[3] B. Borroni, M. Di Luca, A. Padovani, Predicting Alzheimer dementia in mild cognitive impairment patients. Are biomarkers useful? Eur. J. Pharmacol. 545 (2006) 73–80.
[4] C.M. Clark, D. Pratico, L.M. Shaw, S. Leight, S.X. Xie, A. Gu, V.M.-Y. Lee, J.Q. Trojanowski, Commentary on "Optimal design of clinical trials for drugs designed to slow the course of Alzheimer's disease." Biochemical biomarkers of late-life dementia, Alzheimer's & Dementia 2 (2006) 287–293.
[5] X. Shao, E.R. Butch, M.R. Kilbourn, S.E. Snyder, N-[18F]Fluoroethylpiperidinyl, N-[18F]fluoroethylpiperidinemethyl and N-[18F]fluoroethylpyrrolidinyl esters as radiotracers for acetylcholinesterase, Nucl. Med. Biol. 30 (2003) 491–500.
[6] J.P. Blass, Alzheimer's disease and Alzheimer's dementia: distinct but overlapping entities, Neurobiol. Aging 23 (2002) 1077–1084.
[7] S. Akasofu, M. Kimura, T. Kosasa, K. Sawada, H. Ogura, Study of neuroprotection of donepezil, a therapy for Alzheimer's disease, Chem. Biol. Inter. 175 (2008) 222–226.
[8] R.J. Polinsky, Clinical pharmacology of rivastigmine: a new-generation acetylcholinesterase inhibitor for the treatment of Alzheimer's disease, Clin. Ther. 20/4 (1998) 634–647.
[9] J. Coyle, P. Kershaw, Galantamine, a cholinesterase inhibitor that allosterically modulates nicotinic receptors: effects on the course of Alzheimer's disease, Biol. Psychiatry 49/3 (2001) 289–299.
[10] H. Tang, Y.-B. Wei, C. Zhang, F.-X. Ning, W. Qiao, S.-L. Huang, L. Ma, Z.-S. Huang, L.-Q. Gu, Synthesis, biological evaluation and molecular modeling of oxoisoaporphine and oxoaporphine derivatives as new dual inhibitors of acetylcholinesterase/butyrylcholinesterase, Eur. J. Med. Chem. 44 (2009) 2523–2532.
[11] P. Jia, R. Sheng, J. Zhang, L. Fang, Q. He, B. Yang, Y. Hu, Design, synthesis and evaluation of galanthamine derivatives as acetylcholinesterase inhibitors, Eur. J. Med. Chem. 44 (2009) 772–784.
[12] A.A.N.de Paula, J.B.L. Martins, M.L. dos Santos, L.de C. Nascente, L.A.S. Romeiro, T.F.M.A. Areas, K.S.T. Vieira, N.F. Gambo, N.G. Castro, R. Gargano, New potential AChE inhibitor candidates, Eur. J. Med. Chem. 44 (2009) 3754–3759.
[13] G.C. González-Muñoz, M.P. Arce, B. López, C. Pérez, A. Romero, L. del Barrio, D. Martín-de-Saavedra, J. Egea, R. León, M. Villarroya, M.G. López, A.G. García, S. Conde, I. Rodríguez-Franco, N-Acylaminophenothiazines: neuroprotective agents displaying multifunctional activities for a potential treatment of Alzheimer's disease, Eur. J. Med. Chem. 46 (2011) 2224–2235.
[14] B. Tasso, M. Catto, O. Nicolotti, F. Novelli, M. Tonelli, I. Giangreco, L. Pisani, A. Sparatore, V. Boido, A. Carotti, F. Sparatore, Quinolizidinyl derivatives of Bi- and tricyclic systems as potent inhibitors of Acetyl- and butyrylcholinesterase with potential in Alzheimer's disease, Eur. J. Med. Chem. 46 (2011) 2170–2184.
[15] K. Gholivand, Z. Hosseini, S. Farshadian, H. Naderi-Manesh, Synthesis, characterization, oxidative degradation, antibacterial activity and acetylcholinesterase/butyrylcholinesterase inhibitory effects of some new phosphorus(V) hydrazides, Eur. J. Med. Chem. 45 (2010) 5130–5139.
[16] G.L. Ellman, K.D. Courtney, V. Andres Jr., R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7 (1961) 88–95.
[17] W.K. Summers, L.V. Majovski, G.M. Marsh, K. Tachiki, A. Kling, Oral tetrahydroaminoacridine in long treatment of seniledementia, Alzheimer type, N. Engl. J. Med. 315 (1986) 1241–1245.
[18] A. Frideling, R. Faure, J.-P. Galy, A. Kenz, I. Alkorta, J. Elguero, Tetrahydroacridin-9-ones, 9-chlorotetrahydroacridines, 9-amino-tetrahydroacridines and 9-(pyrazol-1-yl)-tetrahydroacridines derived from chiral cyclanones, Eur. J. Med. Chem. 39 (2004) 37–48.
[19] D. dos Santos Pisoni, J. Sobieski da Costa, D. Gamba, C. Liberato Petzhold, A.C. de Amorim Borges, M.A. Ceschi, P. Lunardi, C.A. Saraiva Gonçalves, Synthesis and AChE inhibitory activity of new chiral tetrahydroacridine analogues from terpenic cyclanones, Eur. J. Med. Chem. 45 (2010) 526–535.
[20] M.E. Hadwiger, M. Telting-Diaz, C.E. Lunte, Liquid chromatographic determination of tacrine and its metabolites in rat bile microdialysates, J. Chromatogr. B. Biomed. Appl. 655/2 (1994) 235–241.
[21] L.L. Hansen, J.T. Larsen, K. Brosen, Determination of tacrine and its metabolites in human plasma and urine by high-performance liquid chromatography and fluorescence detection, J. Chromatogr. B. Biomed. Sci. Appl. 712 (1998) 183–191.
[22] D.B. Haughey, C.A. McNaney, M.S. Collis, R.R. Brown, P.H. Siedlik, L. Balogh, P.M. Klockowski, Simultaneous determination of tacrine and 1-hydroxy-, 2-hydroxy-, and 4-hydroxytacrine in human plasma by high-performance liquid chromatography with fluorescence detection, J. Pharm. Sci. 83/11 (1994) 1582–1585.
[23] P. Szymański, E. Mikiciuk-Olasik, Nowe n-alkilo-fluorobenzoilowe pochodne takryny i sposób ich otrzymywania Biul. Urz. Patent(PL), 2010, R 952, no. 13, p. 14.
[24] ICH, Q2(R1) Validation of Analytical Procedures: Text and Methodology., International Conference on Harmonization, Geneva, 2005.
[25] A.J. Bauman, Combining micro dry column chromatography and mass spectrometry (1970).
[26] M.K. Hu, C.F. Lu, A facile synthesis of bis-tacrine isosteres, Tetrah. Lett. 41 (2000) 1815–1818.
[27] Z.J. Kamiński, 2-chloro-4,6-dimetoxy-1,3,5-triazine. A new coupling reagent for peptide synthesis, Synthesis (1987) 917–920.