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International Journal of Pharmaceutical Research & Allied Sciences, 2018, 7(3):233-247

Research Article

ISSN : 2277-3657
CODEN(USA) : IJPRPM

Validation of HPLC Method for Simultaneous Determination of Galantamine Hydrobromide/Pymadine

Dobrina Doncheva Tsvetkova*, Danka Petrova Obreshkova

Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Medical University-Sofia, Bulgaria

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ABSTRACT

In accordance to the perspectives of multi-target therapy of Alzheimer's disease by the combination of acetylcholinesterase inhibitor with a potential synergist, and due to the fact that in literature and pharmacopoeial articles, the methods for the simultaneous analysis of Galantamine and Pymadine haven't been described, the aim of the current investigation was the development of isocratic HPLC method for the simultaneous determination of these drugs in the model mixtures and validation of method for the analytical parameters' selectivity, linearity, LOD, LOQ, accuracy and precision as per ICH requirements.

The chromatographic conditions applied were: column RP C_{18} ODC Spherisorb, column temperature: 25 °C, mobile phase: 50 mM disodium hydrogenphosphate: acetonitrile = 80 : 20 v/v, flow rate: 1.5 ml/min., UV-detection (λ = 280 nm).

The experimental results were subjected to a linear regression analysis. The regression equations obtained demonstrated the linear relationship between the peak area and concentration: $y = 1.10^{10}.x + 387106$ (Galantamine hydrobromide) (LOD = $1.08.10^{-4}$ g/ml; LOQ = $3.6.10^{-4}$ g/ml); $y = 9.10^{9}.x + 1.10^{6}$ (Pymadine) (LOD = $1.32.10^{-4}$ g/ml; LOQ = $4.4.10^{-4}$ g/ml).

Accuracy was represented by the degree of recovery, which data were included in the corresponding confidence interval: 96.66 % ÷ 98.76 % (Galantamine hydrobromide); 99.58 % ÷ 103.86 % (Pymadine). The results for precision suited the relevant interval:

System suitability was confirmed by the lack of statistically significant difference between retention time values: t_R = 3.179 (Galantamine hydrobromide), t_R = 5.272 (Pymadine).

The developed and validated isocratic HPLC method was appropriate for separation, and simultaneoully for identification and determination of Galantamine hydrobromide and Pymadine, for which, the combination HPLC methods haven't been described previously.

Keywords: *HPLC, validation, determination, Galantamine hydrobromide, Pymadine.*

INTRODUCTION

Alzheimer's disease is a chronic progressive polypathogenic neurodegenerative disease [1], in which the combination of various mechanisms and risk factors causes anatomical, cellular and molecular disorders and total disintegration of the intellectual and mental activity [2]. The up-to-date and growing importance of the development of new effective drugs for preventing and slowing down the disease progression is due to the following reasons: 1) a continuous increase of the incidence of the disease; 2) a large number of risk factors; 3) the involvement of various areas of the cerebral cortex: amygdala; frontal, hippocampus, parietal, temporal; 4) a variety of pathogenetic mechanisms of neuronal degeneration; 5) heterogeneous clinical picture, expressed in cognitive deficits, reduced learning ability, behavioral and functional disorders and total disintegration [3].

The classic therapeutic approach for Alzheimer's disease was the compensatory therapy by the reversible acetylcholinesterase inhibitors: Galantamine, Donepezil and Rivastigmine, by suppressing the breakdown of the intrasynaptic neurotransmitter acetylcholine, increase the possibility of conducting a signal to the postsynaptic cholinergic neuron [3]. Free-radical processes damage the biomarker molecules including: proteins, lipids, nucleic acids (DNA, RNA), thereby interfere the intracellular signals and lead to the nerve cell damage and apoptosis, and due to these reasons, they are one of the causes of pathogenetic changes in Alzheimer's disease [4]. A perspective therapeutic trend is multi-drug therapy with compounds containing potential properties for simultaneous responses to the pathogenetic mechanisms of the disease: cholinergic degeneration, amyloid plaques and oxidative stress. In accordance to this trend, the investigations to increase the pharmacological effects by the combination of acetylcholinesterase inhibitor with antioxidant properties of Galantamine (Fig. 1.) with the potential synergist of Pymadine (a drug for the multiple sclerosis treatment) (Fig. 1.) [5], have been ongoing.

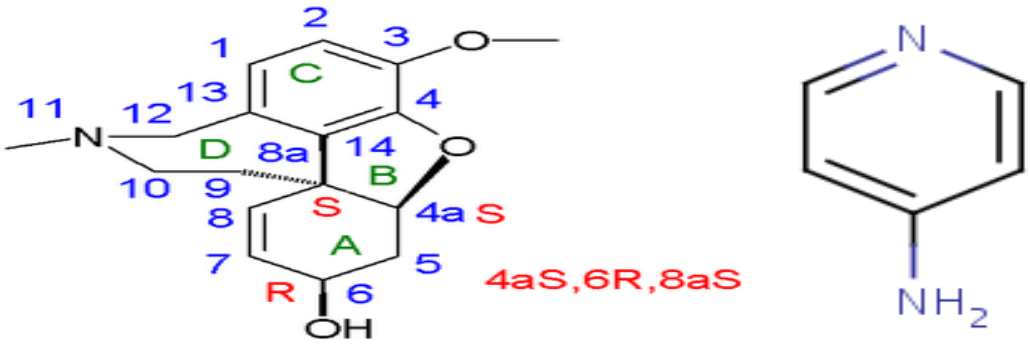


Fig. 1. Chemical stuctures of Galantamine hydrobromide and 4-aminopyridine.

Acetylcholinesterase inhibitor Galantamine [6] leads to the neuroprotection in Alzheimer with cerebrovascular disease [7], vascular dementia and ischemia, due to its antioxidant effects [8]. Galantamine allosterically potentiates α7-nicotinic acetylcholine receptors [9], which is associated with increasing the dopaminergic neurotransmission [10] and the improvement of the processes of learning, memory [9] and attention [11] by being applied alone or in combination with 4-aminopyridine (Pymadine) [12]. Galantamine increases Aβ clearance by the activation of microglia [13].

4-aminopyridine is a drug for the symptomatic treatment of multiple sclerosis [14], which improves walking ability [15] in patients with walking impairment [16] and ameliorates gait [17] in gait disturbances [18].

HPLC methods are often applied for the determination of drugs in human plasma (Glimepiride and Metformin) [19] and in tablet dosage forms of: Deferasirox [20], Telmisartan and Hydrochlorothiazide [21], Olmesartan and Cilnidipine [22] and Amlodipine and Nebivolol [23].

For the analysis of Galantamine hydrobromide in tablets, the following HPLC methods have been described: 1) HPLC with UV-detection at λ = 285 nm [24] and λ = 288 nm [25]; 2) HPLC with a fluorescence detection at λexcitation = 290 nm and λemmision = 320 nm, on a Venusil XBP RP C_{18} column, mobile phase: methanol : 25 mM sodium dihydrogenphosphate = 84 : 16 v/v and flow rate: 1.0 ml/min. [26]. For determination of 4-aminopyridine in capsules, HPLC method was developed at the isocratic mode at λ = 263 nm [27].

In accordance to the perspectives of multi-target therapy of Alzheimer's disease by the combination of acetylcholinesterase inhibitor with its potential synergist, and due to the fact that the methods for simultaneous analysis of the components existing in the combination of Galantamine/Pymadine haven't been described in the literature and pharmacopoeial articles, the aim of the current investigation was the development and validation as per ICH requirements of the HPLC method for the simultaneous determination of Galantamine hydrobromide and Pymadine in model mixtures.

MATERIALS

I. Pharmacopoeial purity compounds which were investigated: Galantamine hydrobromide (Sopharma, N: 10796132); 4-aminopyridine.

II. Reagents with pharmacopoeal purity: disodium hydrogenphosphate (99.5 %) (Merck, N: K28661174105).

III. Solvents with pharmacopoeial purity: acetonitrile (99.9 %) (Sigma Aldrich, N: SZBD 150 SV UN 1648), methanol (99.9 %) (Sigma Aldrich, N: SZBD 063AV UN 1230), distilled water.

Methods: HPLC method for simultaneous determination of Galantamine hydrobromide and Pymadine in model mixtures

I. Equipmentt

The chromatography was carried out by using HPLC system of Shimadzu LC-10 Advp Liquid Chromatograph, and fixed-length wavelength SPD 10 AVP UV-VIS detector.

II. Chromatographic conditions

The chromatographic conditions applied were: the isocratic mode, the stationary phase: column RP C_{18} ODC Spherisorb (250 mm × 4.6 mm × 5 μm), the column temperature: 25 °C, mobile phase: 50 mM disodium hydrogenphosphate : acetonitrile = 80 : 20 v/v, the flow rate: 1.5 ml/min., the UV-detection at the analytical wavelength of λ = 280 nm. The volume of the injection loop was 20 μl prior to the injection of the drug solution. The column was equilibrated for at least 30 min, with the mobile phase following through the system. The mobile phase was degassed.

III. Preparation of solutions of reference substances of Galantamine hydrobromide and 4-aminopyridine for the validation of HPLC method with respect to the analytical parameter linearity

From reference substance Galantamine hydrobromide, the accurately measured quantities of: 0.001 g, 0.002 g, 0.003 g, 0.005 g, 0.01 g, 0.02 g, 0.03 g, 0.07 g, 0.09 g, 0.1 g, were separately dissolved in distilled water in 100.0 ml volumetric flasks, to obtain solutions with the the following concentrations: 1.10^{-5} g/ml, 2.10^{-5} g/ml, 3.10^{-5} g/ml, 5.10^{-5} g/ml, 1.10^{-4} g/ml, 2.10^{-4} g/ml, 3.10^{-4} g/ml, 7.10^{-4} g/ml, 9.10^{-4} g/ml, 1.10^{-3} g/ml.

From reference substances of 4-aminopyridine, the accurately measured quantities of: 0.005 g, 0.01 g, 0.05 g, 0.07 g, 0.08 g, 0.09 g were separately dissolved in distilled water in 100.0 ml volumetric flasks, to obtain solutions with the following concentrations: 5.10^{-5} g/ml, 1.10^{-4} g/ml, 5.10^{-4} g/ml, 7.10^{-4} g/ml, 8.10^{-4} g/ml, 9.10^{-4} g/ml.

IV. Preparation of model mixtures of the reference substances of Galantamine hydrobromide and 4-aminopyridine for the validation of HPLC method with respect to the analytical parameters' accuracy and internal precision (repeatability)

6 model mixtures were prepared by the following manner from the accurately measured quantities of the reference substances: 0.1 g Galantamine hydrobromide and 0.05 g 4-aminopyridine were dissolved in distilled water in 100.0 ml volumetric flasks.

V. Root mean square error method (RMSE) for the determination of the limit of detection (LOD) and the limit of quantitation (LOQ)

The calibration curves were constructed by the analysis of solutions with absorbance A < 0.2. The data were subjected to the linear regression analysis, and the linear correlation coefficients (R^2) were obtained. From the regression equation: y = a.x + b, the predictable absorbance value (Ap); error E = |Ap - A|; E2 = $[|Ap - A|]^2$, E1= $\frac{\sum E2}{n-2}$; RMSE = $\sqrt{E1}$; LOD = 3.RMSE/a; LOQ = 10.RMSE/a, were calculated [28].

RESULTS AND DISCUSSION

HPLC method for the quantification of Galantamine hydrobromide and Pymadine in model mixtures was validated for the analytical validation parameters' specificity, linearity, limit of detection, limit of quantitation, accuracy and internal precision (repeatability) according to International Conference on Harmonization (ICH) guidelines [29].

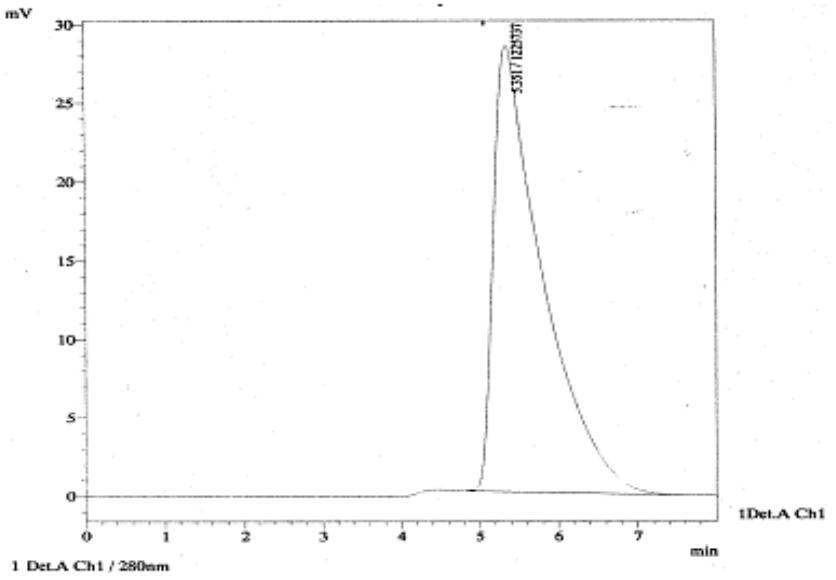
I. Investigation of the analytical parameters' selectivity

In the same manner like solutions with reference standards such as Galantamine hydrobromide and Pymadine, a blank solution containing only a solvent was prepared, and chromatographed for the estimation of the analytical parameters' selectivity, which was confirmed by the fact that in the blank stolution, no retention times (t_R) were observedcorresponding to the retention time for Galantamine hydrobromide ($\overline{X}$ t_R = 3.179) and Pymadine ($\overline{X}$ t_R = 5.272).

II. Investigation of the analytical parameters' linearity, limit of detection and limit of quantitation

Linearity is the range within the signal from the detector which remains in the linear dependency on the concentration of analyte [29]. For analysis of the analytical parameter linearity for the standard solutions with increasing concentration of Galantamine hydrobromide (1.10^{-5} g/ml ÷ 1.10^{-3} g/ml) and 4-aminopyridine (5.10^{-5} g/ml ÷ 9.10^{-4} g/ml), the relationship between the peak area and concentration was investigated. Chromatograms of standard solutions of Galantamine hydrobromide have been illiustrated in Fig. 2. (5.10^{-5} g/ml, 2.10^{-4} g/ml). Chromatograms of standard solutions of 4-aminopyridine have been shown in Fig. 3. (8.10^{-4} g/ml, 9.10^{-4} g/ml).

5.10^{-5} g/ml

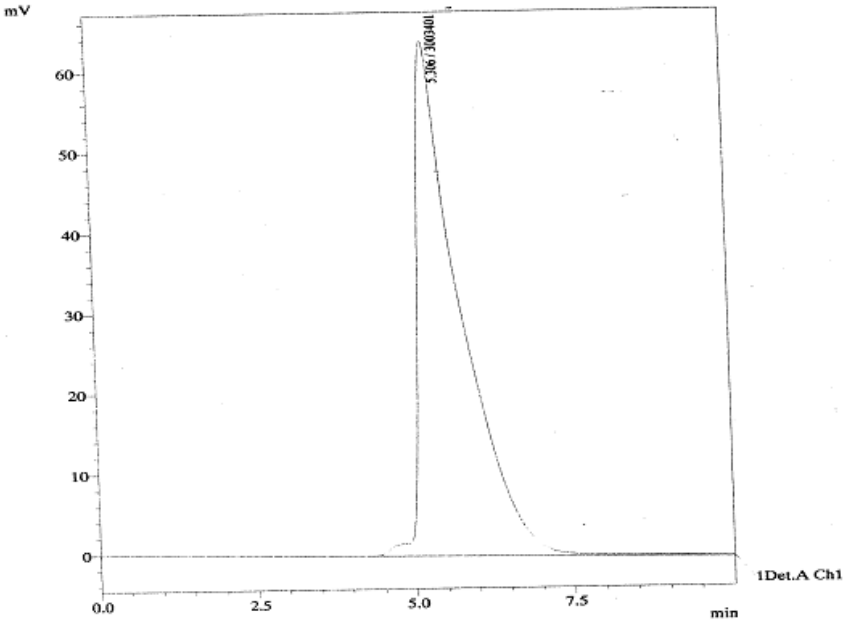


PeakTable

Detector A Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	5.351	1225737	28371	100.000	100.000
Total		1225737	28371	100.000	100.000

2.10^{-4} g/ml



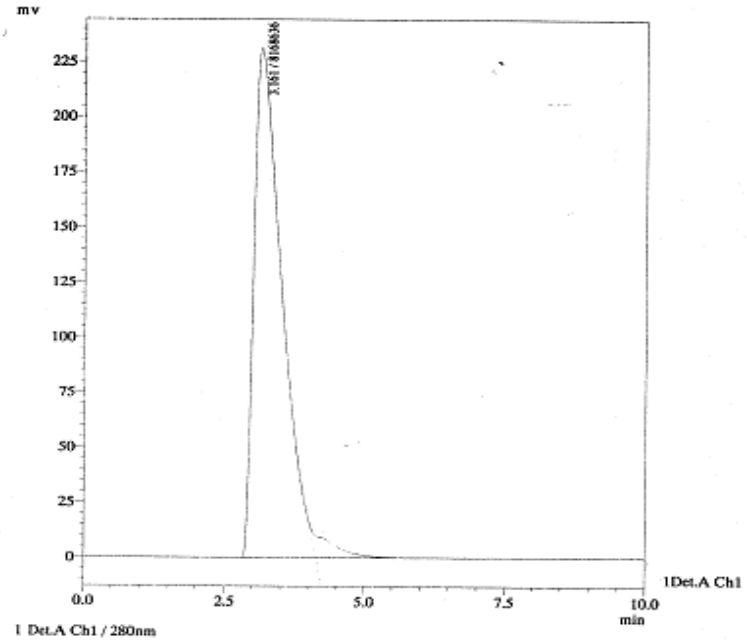
PeakTable

Detector A Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	5.306	3003401	64042	100.000	100.000
Total		3003401	64042	100.000	100.000

Fig. 2. Chromatograms of standard solutions of Galantamine hydrobromide (5.10^{-5} g/ml, 2.10^{-4} g/ml)

8.10^{-4} g/ml

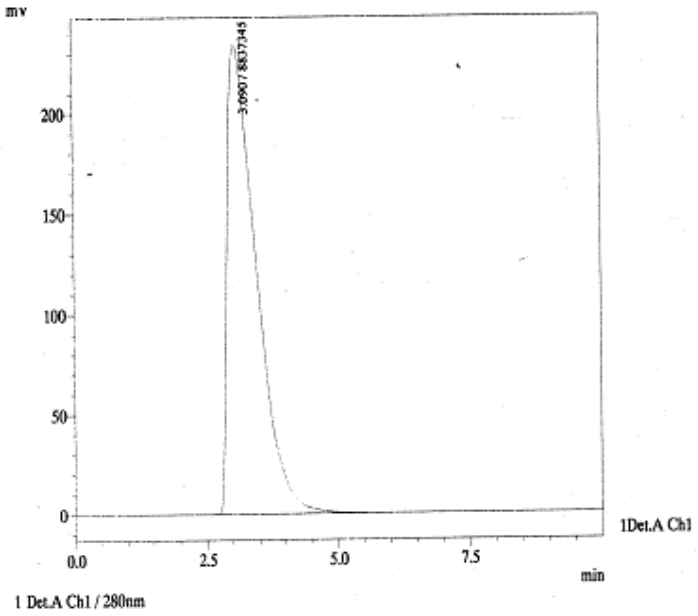


PeakTable

Detector A Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.161	8168636	231244	100.000	100.000
Total		8168636	231244	100.000	100.000

9.10^{-4} g/ml



PeakTable

Detector A Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.090	8837345	234716	100.000	100.000
Total		8837345	234716	100.000	100.000

Fig. 3. Chromatograms of standard solutions of 4-aminopyridine (Pymadine) (8.10^{-4} g/ml, 9.10^{-4} g/ml)

In Table 1, HPLC parameters for standard solutions of Galantamine hydrobromide and 4-aminopyridine have been presented: t_R [min.] – retention time, $w_{0.05}$ [mm] – peak width at 5 % of peak height, T – tailing factor, f – distance from the beginning of the peak to the intersection of the peak height with the peak width at 5 % of the peak height.

Table 1. HPLC parameters for standard solutions of Galantamine hydrobromide and 4-aminopyridine

HPLC parameter	Standard solutions of 4-aminopyridine							Standard solutions of Galantamine hydrobromide				
	1	2	3	4	5	6	7	1	2	3	4	5
t_R [min.]	3.090	3.161	3.181	2.919	3.300	3.495	3.143	5.176	5.306	5.338	5.351	5.329
$w_{0.05}$ [mm]	12	11	12.5	12	11.5	10.5	10.5	12.5	13	13.5	13	12
$T = \frac{w\,0.05}{2f}$	1	1	1.04	1	1.05	1.05	1.05	1	1	0.84	0.84	0.83

The experimental results were subjected to linear regression analysis. The resulting calibration curves showed the relationship between the peak area (A) and the concentration C [g/ml], which have been illustrated in Fig. 4.

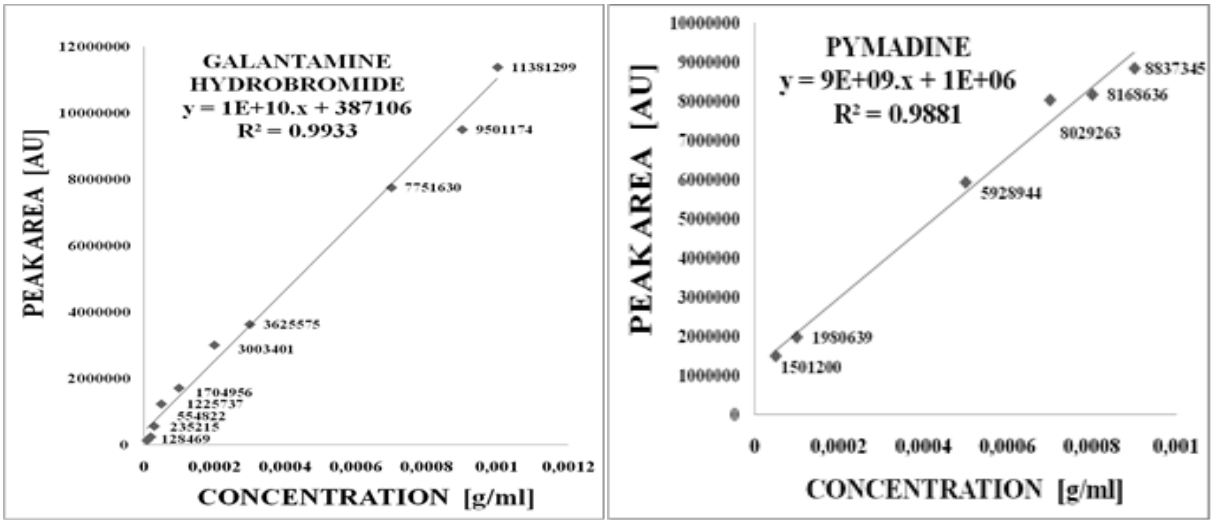


Fig. 4. Calibration curves for Galantamine hydrobromide and Pymadine

Linearity is characterized by the calculated linear correlation coefficients (R^2), which are higher than 0.988. The results of the analysis of the standard solutions with increasing concentration of Galantamine hydrobromide (Table 2.) and 4-aminopyridine (Table 3.) for the investigation of linearity, LOD and LOQ have been summarized as follows: C [g/ml] – concentration, A – measured peak area, Ap – calculated by calibration curve peak area, RMSE = root mean square error, LOD = 3.RMSE/a, LOQ = 10.RMSE/a [28]. The calculation of LOD and LOQ was based on the regression equations: $y = 1.10^{10}.x + 387106$ (Galantamine hydrobromide) and $y = 9.10^{9}.x + 1.10^{6}$ (Pymadine) (Fig. 4.), which demonstrated the linear relationship between the peak area (A) and the concentration C [g/ml] at the corresponding concentration intervals of: 1.10^{-5} g/ml ÷ 1.10^{-3} g/ml (Galantamine hydrobromide); 5.10^{-5} g/ml ÷ 9.10^{-4} g/ml (Pymadine).

Table 2. Investigation of analytical parameters' linearity, LOD and LOQ for Galantamine hydrobromide

N:	C [g/ml]	A	A_p	$E = \|A_p - A\|$	$E^2 = [\|A_p - A\|]^2$
1.	1.10^{-5}	128469	493575	365106	133302391236
2.	2.10^{-5}	235215	600044	364829	133100199241
3.	3.10^{-5}	554822	706513	151691	23010159481
4.	5.10^{-5}	1225737	919450	306287	93811726369
5.	1.10^{-4}	1704956	1451795	253161	64090491921
6.	2.10^{-4}	3003401	2516485	486916	237087191056
7.	3.10^{-4}	3625575	3581174	44401	1971448801
8.	7.10^{-4}	7751630	7839932	88302	7797243204
9.	9.10^{-4}	9501174	9969311	468137	219152250769
10.	1.10^{-3}	11381299	11034000	347299	120616595401
$\sum E2 = 1033939697479$		$E1 = \frac{\sum E2}{n-2} = 129242462185$		$RMSE = \sqrt{129242462185} = 359503$	
$LOD = (3.359503)/1.10^{10} = 1.08.10^{-4}$ g/ml			$LOQ = (10.359503)/1.10^{10} = 3.6.10^{-4}$ g/ml		

Table 3. Investigation of analytical parameter linearity, LOD and LOQ for 4-aminopyridine

N:	C [g/ml]	A	A_p	$E = \|A_p - A\|$	$E^2 = [\|A_p - A\|]^2$
1.	5.10^{-5}	1501200	1638647	137447	18891677809
2.	1.10^{-4}	1980639	2086177	105538	11138269444
3	5.10^{-4}	5928944	5666416	262528	68920950784
4.	7.10^{-4}	8029263	7456536	572727	328016216529
5.	8.10^{-4}	8168636	8351595	182959	33473995681
6.	9.10^{-4}	8837345	9246655	409310	167534676100
$\sum E2 = 627975786347$		$E1 = \frac{\sum E2}{n-2} = 156993946586.75$		$RMSE = \sqrt{156993946586.75} = 396225$	
$LOD = (3.396225)/9.10^{9} = 1.32.10^{-4}$ g/ml			$LOQ = (10.396225)/9.10^{9} = 4.4.10^{-4}$ g/ml		

Parameters of regression equations have been described in Table 4.

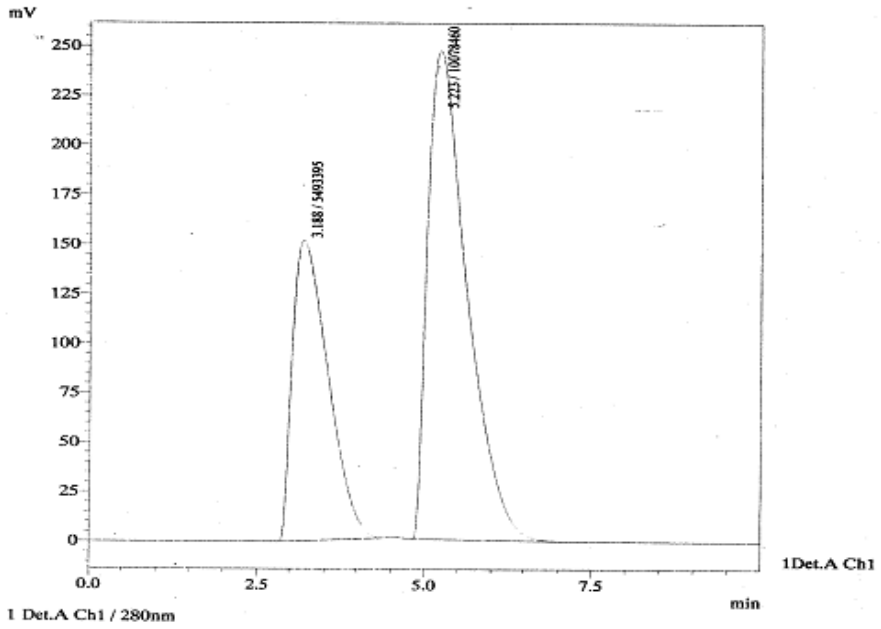
Table 4. Parameters of regression equations for Galantamine hydrobromide and Pymadine

N:	Parameter	Galantamine hydrobromide	Pymadine
1.	Linear interval [g/ml]	$1.10^{-5} \div 1.10^{-3}$	$5.10^{-5} \div 9.10^{-4}$
2.	Regression equation	$y = 1.10^{10}.x + 387106$	$y = 9.10^{9}.x + 1.10^{6}$
3.	Slope (a)	1.10^{10}	9.10^{9}
4.	Standard slope error	3.10^{8}	5.10^{8}
5.	Intersept (b)	387106	1.10^{6}
6.	Standard intersept error	153057	3.10^{5}
7.	Correlation coefficient (R^2)	0.9933	0.9881

III. Investigation of the analytical parameters' accuracy and internal precision (repeatability) for the model mixtures of Galantamine hydrobromide and Pymadine.

6 model mixtures containing 10 mg of Galantamine hydrobromide and 5 mg of Pymadine were analyzed by the HPLC method in a chromatographic system with : stationary phase of: RP (250 mm × 4.6 mm × 5 μm); column temperature of: 25 ºC; mobile phase of : 80 : 20 v/v = 50 mM disodium hydrogenphosphate : acetonitrile; flow rate of : 1 ml/min.; the isocratic mode and UV-detection at λ = 280 nm. Chromatograms of the model mixtures have been illustrated in Fig. 5. (Model Mixtures 1 and 2), Fig. 6. (Model Mixtures 3 and 4) and Fig. 7. (Model Mixtures 5 and 6).

Model mixture 1

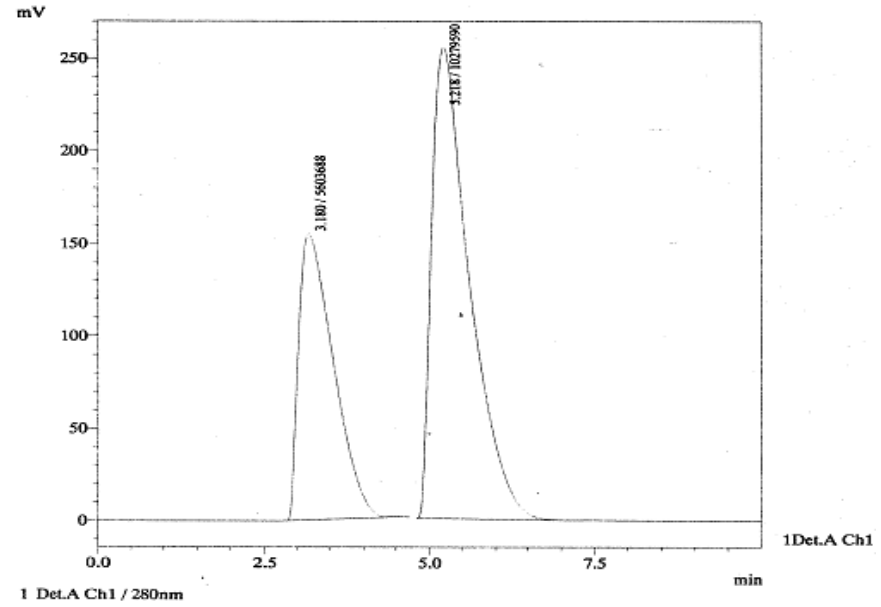


PeakTable

Detector A Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.188	5493395	151586	35.278	38.051
2	5.223	10078460	246787	64.722	61.949
Total		15571855	398374	100.000	100.000

Model mixture 2



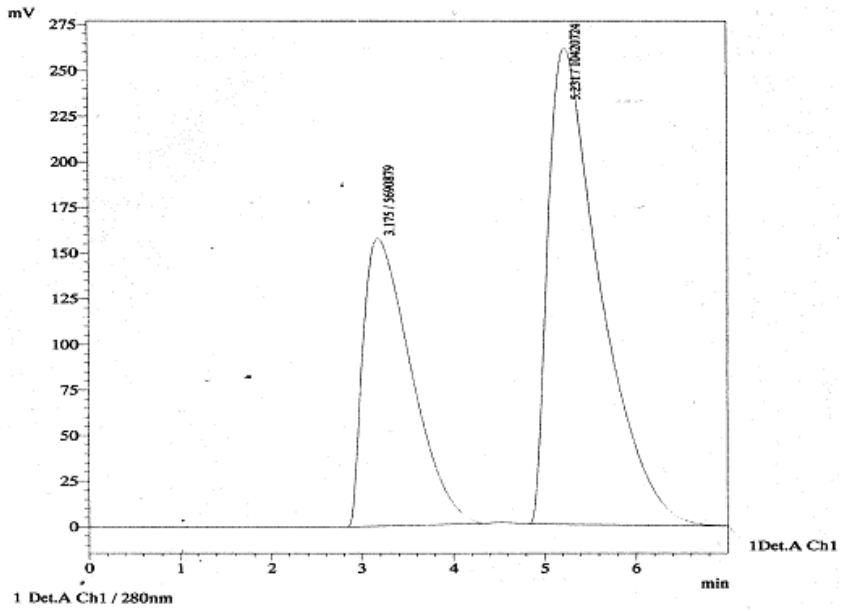
PeakTable

Detector A Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.180	5603688	154778	35.280	37.809
2	5.218	10279590	254596	64.720	62.191
Total		15883278	409374	100.000	100.000

Fig. 5. Chromatograms of model mixtures 1 and 2, containing Galantamine hydrobromide and Pymadine at $\lambda = 280$ nm

Model mixture 3

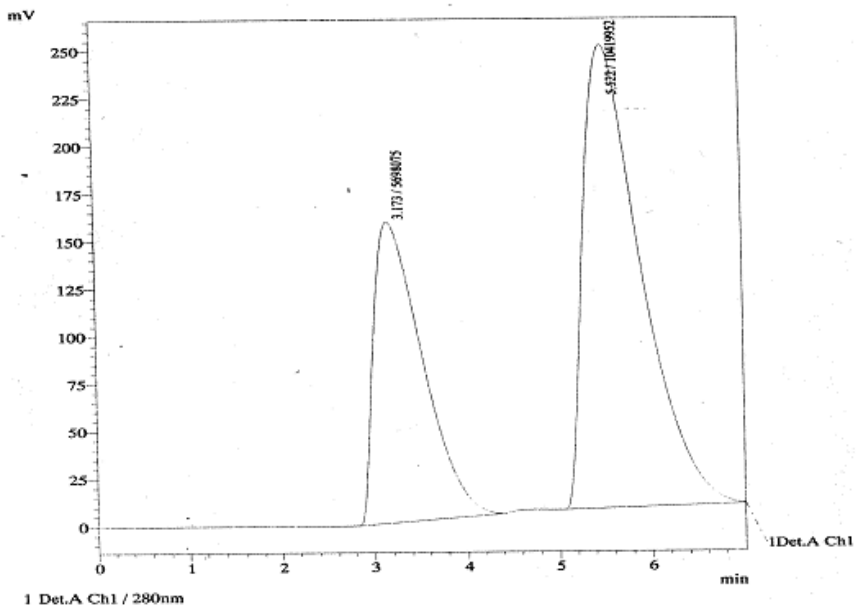


PeakTable

Detector A Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.175	5690879	157671	35.322	37.652
2	5.231	10420724	261093	64.678	62.348
Total		16111603	418765	100.000	100.000

Model mixture 4



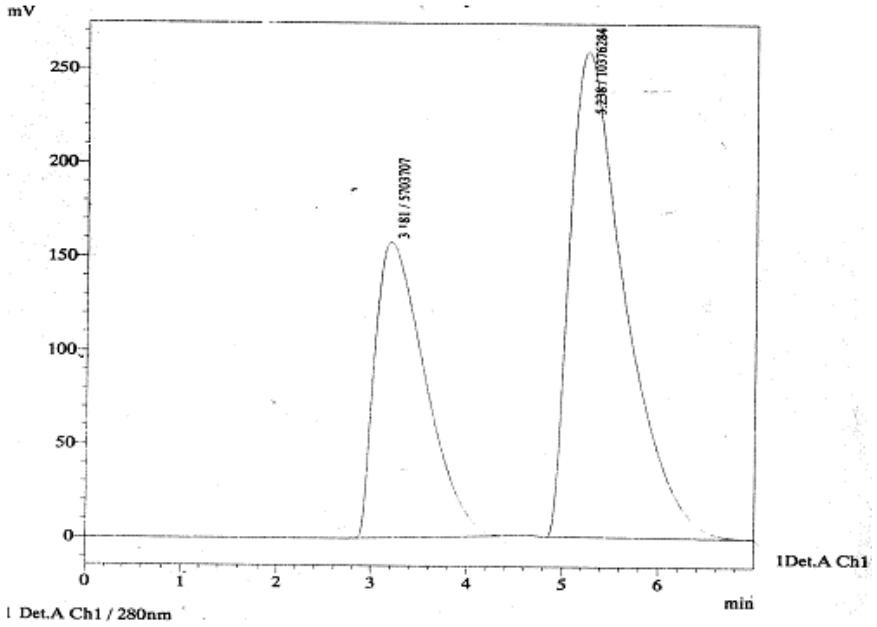
PeakTable

Detector A Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.173	5698075	158183	35.352	39.390
2	5.522	10419952	243395	64.648	60.610
Total		16118026	401578	100.000	100.000

Fig. 6. Chromatograms of model mixtures 3 and 4, containing Galantamine hydrobromide and Pymadine at $\lambda = 280$ nm

Model mixture 5

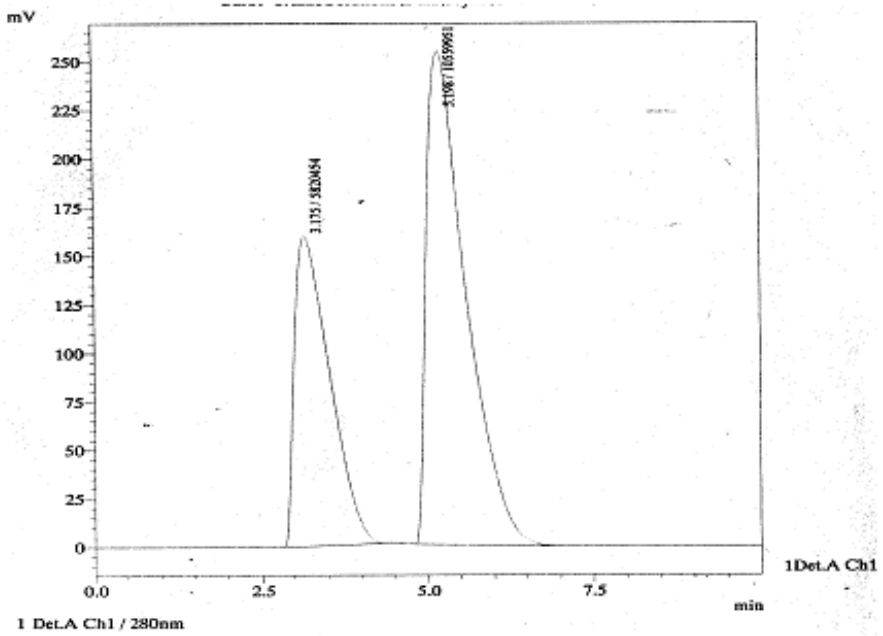


PeakTable

Detector A Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.181	5703707	157930	35.471	37.872
2	5.238	10376284	259077	64.529	62.128
Total		16079991	417007	100.000	100.000

Model mixture 6



PeakTable

Detector A Ch1 280nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	3.175	5820454	159637	35.533	38.591
2	5.198	10559951	254030	64.467	61.409
Total		16380405	413666	100.000	100.000

Fig. 7. Chromatograms of model mixtures 5 and 6, containing Galantamine hydrobromide and Pymadine at $\lambda = 280$ nm

The HPLC parameters for the model mixtures have been included in Table 5 : t_R – retention time, k' – capacitance factor, α – selectivity, Rs – degree of separation, $w_{0.05}$ [mm] – peak width at 5 % of peak height, T – tailing factor, f – distance from the beginning of the peak to the intersection of the peak height with the peak width at 5 % of the peak height.

Table 5. HPLC-parameters for model mixtures of Galantamine hydrobromide and Pymadine

HPLC para-meter	Model mixture 1		Model mixture 2		Model mixture 3		Model mixture 4		Model mixture 5		Model mixture 6	
	P	G	P	G	P	G	P	G	P	G	P	G
t_R [min.]	3.188	5.223	3.180	5.218	3.175	5.231	3.173	5.522	3.181	5.238	3.175	5.198
k′	0.096	0.795	0.093	0.793	0.091	0.796	0.090	0.898	0.093	0.800	0.091	0.786
α	8.28		8.53		8.75		9.98		8.60		8.64	
Rs	1.77		1.74		1.88		2.06		2.00		1.63	
$w_{0.05}$ [mm]	12	12	12	13	15	17.5	15.5	19	13	17	12	13
T = $\frac{w\ 0.05}{2f}$	1	1.17	1	1.17	1.13	1	0.9	1	1	1	1.17	1.17

The model mixtures of Galantamine hydrobromide (G) and Pymadine (P) have been demonstrated in Table 6, the results have been summarized as follows:

1) the peak area (A): A G_{10}, A P_5;
2) Chauvenet's criterion for the peak area (U A): U A G_{10}, U A P_5;
3) the peak height (H): H G_{10}, H P_5;
4) Chauvenet's criterion for the peak height (U H): U H G_{10}, U H P_5.

Table 6. Peak area and peak height for Galantamine hydrobromide and Pymadine

N:	A P_5	U A P_5	H P_5	U H P_5	A G_{10}	U AG_{10}	H G_{10}	U H G_{10}
1.	5493395	1.59	151586	1.72	10078460	1.7	246787	0.93
2.	5603688	0.59	154778	0.63	10279590	0.47	254596	0.21
3.	5690879	0.20	157671	0.35	10420724	0.4	261093	1.15
4.	5698075	0.27	158183	0.53	10419952	0.39	243395	1.42
5.	5703707	0.32	157930	0.44	10376284	0.13	259077	0.86
6.	5820454	1.38	159637	1.02	10559951	1.25	254030	0.13
$\overline{X}$ ± SD	5668366		156631		1035827		253163	
SD	110061		2936		163189		6881	
RSD	1.94		1.87		1.58			

By the calibration curve method, the amount of Galantamine hydrobromide [G_{10}] and Pymadine [P_5] was calculated in each model mixture, using the peak area of the components of the respective chromatograms of the model mixtures.

In Table 7, the results have been presented for: N – number of measurements (1 ÷ 6); [G_{10}], [P_5] – quantity of components in model mixtures; R [G_{10}], R [P_5] – degree of recovery [%]; U [G_{10}], U [P_5] – the criterion of Chauvenet's, [P] – the amount of components incorporated in the model mixtures; $\overline{X}$ – mean arithmetic value; SD – standard deviation; RSD [%] – related standard deviation; $S_{\overline{X}}$ – mean square error; P – confidence probability [%]; t – coefficient of Student; $\overline{X} \pm t.S_{\overline{X}}$ – confidence interval; E – relative error [%].

Table 7. Content of Galantamine hydrobromide and Pymadine model mixtures and degree of recovery**.**

N:	[P_5] [mg/10 ml]	R[P_5] [%]	U [P_5]	[P] [mg/10 ml]	[G_{10}] [mg/10 ml]	R[G_{10}] [%]	U [G_{10}]	[G] [mg/10 ml]
1.	4.99	99.8	1.47	5.0	9.69	96.9	1.73	10.0
2.	5.12	100.39	1.02	5.1	9.89	96.96	0.5	10.2
3.	5.21	102.16	0.34	5.1	10.03	98.33	0.38	10.2
4.	5.22	102.35	0.48	5.1	10.03	98.33	0.38	10.2
5.	5.23	102.55	0.63	5.1	9.99	97.94	0.13	10.2
6.	5.36	103.08	1.04	5.2	10.17	97.79	1.25	10.4
$\overline{X}$ ± SD	5.19 ± 0.12				9.97 ± 0.16			
$\bar{R}$ [%] ± RSD [%]		101.72 ± 1.29				97.71 ± 0.65		
SD	0.12	1.31			0.16	0.64		
RSD [%]	2.31	1.29			1.6	0.65		
S $\overline{X}$	0.05	0.53			0.07	0.26		
P [%]	99.0	99.0			99.0	99.0		
t	4.03	4.03			4.03	4.03		
t.S $\overline{X}$	0.2	2.14			0.28	1.05		
$\overline{X}$ – t.S $\overline{X}$ ÷ $\overline{X}$ + t.S $\overline{X}$	4.99 ÷ 5.39	99.58 ÷ 103.86			9.69 ÷ 10.25	96.66 ÷ 98.76		
E [%]	0.96	0.52			0.7	0.27		

Analytical parameter accuracy is the degree of correspondence between the obtained average results of the repeated analysis and the actual value [29]. Accuracy has been represented by the degree of recovery R [%] ± RSD [%] as per ICH guidelines. [29]: R [%] ± RSD [%]: 97.71 % ± 0.65 % (Galantamine hydrobromide); 101.72 % ± 1.29 % (Pymadine). All the experimental data for R [%] were included in the corresponding confidence interval with the confidence probability of P = 99.0 % (t = 4.03): 96.66 % ÷ 98.76 % (Galantamine hydrobromide); 99.58 % ÷ 103.86 % (Pymadine). For Galantamine hydrobromide SD and RSD were lower than 0.7, and for Pymadine, SD and RSD were lower than 1.4 (Table 7.).

For the assessment of the need for the removal of sharply different results, Chauvenet's criterion was applied [29]. Chauvenet's criterions were lower than the standard maximum value, which indicated that no statistically significant difference was observed between the obtained amounts of the compounds in the homogeneous assay analysis.

IV. Precision (repeatability)

Analytical parameter repeatability has been characterized by the uncertainty of the results, which included standard deviation (SD), relative standard deviation (RSD) and the confidential interval ($\overline{X}$ ± t.S $\overline{X}$) [29, 30]. For the investigation of the repeatability of the data for the peak area, and for the obtained quantities of Galantamine hydrobromide and Pymadine for 6 model mixtures, SD, RSD and confidence interval were estimated by which the repeatability was characterized.

The experimental results showed that at the confidence possibility of P = 99.0 % (t = 4.03), all the data for the content of the components of the mixtures suited the corresponding confidence interval: 9.69 mg ÷ 10.25 mg (Galantamine hydrobromide) and 4.99 mg ÷ 5.39 mg Pymadine) at SD < 0.2. The values of the Chauvenet's criterion were lower than the standard Umax = 1.73 (N = 6), which confirmed that no statistically significant difference between the obtained quantities in the analysis of 6 homogeneous samples was observed (Table 7.).

V. System suitability test

The system suitability test was carried out by following the retention times in the analysis of 6 model mixtures. The retention times of the components in the model mixtures have been included in Table 8.

Table 8. Retention times t_R [min.] for Galantamine hydrobromide and Pymadine in model mixtures

N:	Galantamine hydrobromide	Pymadine
	t_R [min.]	t_R [min.]
1.	3.188	5.223
2.	3.180	5.218
3.	3.175	5.231
4.	3.173	5.522
5.	3.181	5.238
6.	3.175	5.198
$\overline{X}$ ± SD	3.179 ÷ 0.006	5.272 ÷ 0.12
RSD [%]	0.19	2.28

The system suitability was confirmed by the lack of statistically significant difference between the values of the chromatographic parameter retention time t_R [min.] for the components in the analysis of 6 samples: $\overline{X}$ t_R = 3.179 (Galantamine hydrobromide) and $\overline{X}$ t_R = 5.272 (Pymadine) (Table 8.).

For determination of Galantamine in human plasma and urine, HPLC method based on the derivatization with 5-(dimethylamino)naphthalene-1-sulfonylchloride (dansyl chloride) and fluorescence detection at λexcitation = 375 nm, λemission = 537 nm have been described [31].

In comparison with this method, the advantage of the current HPLC method was the analysis of Galantamine without derivatization reaction.

First order derivative method for the assay of Galantamine hydrobromide in tablet at zero crossing point λ = 286.4 nm [32] and λ= 277.4. nm [33] has been reported.

The disadvantage of derivative spectrophotometry has been its susceptibility towards changes in the apparatus parameters. Small differences in the wavelength setting have had a great effect on the result, especially in the zero-crossing technique, where errors in the registration of the spectrum have been the reasons for the method's non-reproducibility. In comparison with the derivative spectrophotometry, the advantage of HPLC has been its low susceptibility towards changes in the apparatus parameters [34], and the advantage of the current HPLC method was that it provided quick analysis with high resolution, accuracy and precision not only for Galantamine hydrobromide, but for its separation and determination in combination with Pymadine.

CONCLUSION

In accordance to the fact that in literature and pharmacopoeial articles, the methods for the simultaneous analysis of Galantamine and Pymadine haven't been described, the advantage of the current work was that the new method for the identification and determination of those drugs in combination was developed and validated for the analytical parameters' selectivity, linearity, LOD, LOQ, accuracy and precision as per ICH requirements.

The specificity of the method was confirmed by the absence of peaks with retention times corresponding with those of drugs. The regression equations obtained demonstrated the linear relationship between the concentrations of Galantamine hydrobromide LOD = $1.08.10^{-4}$ g/ml; LOQ = $3.6.10^{-4}$ g/ml., and of Pymadine LOD = $1.32.10^{-4}$ g/ml; LOQ = $4.4.10^{-4}$ g/ml. All the experimental data for the degree of recovery were included in the corresponding confidence interval: 96.66 % ÷ 98.76 % (Galantamine hydrobromide); 99.58 % ÷ 103.86 % (Pymadine). The method ensured that accuracy of SD and RSD was lower than 1.5, and the repeatability of SD and RSD was lower than 0.2.

The lack of statistically significant difference between the values of the chromatographic parameter retention time for Galantamine hydrobromide and Pymadine proved the system's suitability for the developed HPLC method.

Chauvenet's criterions are lower than the standard maximum value, which indicated that no statistically significant difference was observed between the obtained amounts of compounds in the homogeneous assay analysis.
The developed and validated isocratic HPLC method was appropriate for separation, and simultaneously identification and determination of Galantamine hydrobromide and Pymadine, for which the combination of HPLC methods haven't been described previously.

CONFLICTS OF INTERESTS
All authors had none to declare

REFERENCES

1. Nikolova, I., Danchev, N., Lamvob, N., Grudeva V. Neurodegenerative diseases in geriatry – opportunities of current pharmacotherapy. Clin. Homeopathy, 2010, 1(1), 3-6.
2. Knobloch, M., Mansuy, I.M. Dendritic spine loss and synaptic alterations in Alzheimer's disease. Mol. Neurobiol., 2008, 37(1), 73-82.
3. Danchev, N., Nikolova, I. Pharmacological treatment of cognitive impairements in Alzheimer's disease. Autonomic Autocoid Pharmacol., 2006, 26(1), 46-49.
4. Ansari, M.A., Scheff, S.W. Oxidative stress in the progression of Alzheimer disease in the frontal cortex. J. Neuropathol. Exp. Neurol., 2010, 69(2), 155-167.
5. Pikoulas, T.E., Fuller, M.A. Dalfampridine: a medication to improve walking in patients with multiple sclerosis. Ann. Pharmacother., 2012, 46(7-8), 1010-1015.
6. Ago, Y., Koda, K., Takuma, K., Matsuda, T. Pharmacological aspects of the acetylcholine-esterase inhibitor Galantamine. J. Pharmacol. Sci., 2011, 116(1), 6-17.
7. Zarotsky, V., Sramek, J.J., Cutler, N.R. Galantamine hydrobromide: an agent for Alzheimer's disease. Amer. J. Health System Pharm., 2003, 60(5), 446-452.
8. Erkinjuntti, T., Kurz, A., Small, G.W., Bullock, R., Lilienfeld, S., Damaraju, C.V. An open-label extension trial of Galantamine in patients with probable vascular dementia and mixed dementia. Clin. Ther., 2003, 25(6), 1765-1782.
9. Woodruff-Pak, D.S., Vogel, R.W., Wenk, G.L. Galantamine: effect on nicotinic receptor binding, acetylcholinesterase inhibition and learning. Proc. Natl. Acad. Sci. USA, 2001, 98(4), 2089-2094.
10. Galvin, J.E., Cornblatt, B., Newhouse, P., Ancoli-Israel, S., Wesnes, K., Williamson, D., Zhu, Y., Sorra, K., Amatniek, J. Effects of Galantamine on measures of attention: results from 2 clinical trials in Alzheimer disease patients with comparisons to Donepezil. Alzheimer Dis. Assoc. Disord., 2008, 22(1), 30-38.
11. Vellas, B., Cunha, L., Gertz, H.J., De Deyn, P.P., Wesnes, K., Hammond, G., Schwalen, S. Early onset effects of Galantamine treatment on attention in patients with Alzheimer's disease. Curr. Med. Res. Opin., 2005, 21(9), 1423-1429.
12. Markov M., Danchev N., Uzunov P., Higashino H., Suzuki A. Influence of Nivalin P on the training and memorizing processes in rats. Acta Med. Kinki. Univ., 1994, 19(2), 119-126.
13. Takata, K., Kitamura, Y., Saeki, M., Terada, M., Kagitani, S., Kitamura, R., Fujikawa, Y., Maelicke, A., Tomimoto, H., Taniguchi, T., Shimohama, S. Galantamine-induced amyloid-β clearance mediated via stimulation of microglial nicotinic acetylcholine receptors. J. Biol. Chem., 2010, 285(51), 40180-40191.
14. Jensen, H.B., Ravnborg, M., Dalgas, U., Stenager, E. 4-aminopyridine for symptomatic treatment of multiple sclerosis: a systematic review. Ther. Adv. Neurol. Disord., 2014, 7(2), 97-113.
15. Hayes, K.C. Impact of extended-release Dalfampridine on walking ability in patients with multiple sclerosis. Neuropsychiatr. Dis. Treat., 2011, 7(1), 229-239.
16. Hersh, C., Rae-Grant, A. Extended-release Dalfampridine in the management of multiple sclerosis-related walking impairment. Ther. Adv. Neurol. Disord., 2012, 5(4), 199-204.
17. Sahraian, M.A., Maghzi, A.H., Etemadifar, M., Minagar, A. Dalfampridine: review of its efficacy in improving gait in patients with multiple sclerosis. J. Cent. Nerv. Syst. Dis., 2011, 16(3), 87-93.
18. Egeberg, M.D., Oh, C.Y., Bainbridge, J.L. Clinical overview of Dalfampridine: an agent with a novel mechanism of action to help with gait disturbances. Clin. Ther., 2012, 34(11), 2185-2194.

19. Donia, A.R.M.A., Abdel-Gawad, S,A., Al-Kahtani, A.A., Imam, F. UPLC-MS/MS method for simultaneous quantification of Glimepiride and Metformin in human plasma. Int. J. Pharm. Res. Allied Sci., 2017, 6(4), 80-87.
20. Kothawade, J.R., Kapil, M., Agrawal, K.M., Lad V.S. Stability indicating RP-HPLC assay method development and validation for determination of Deferasirox in tablet dosage form. Int. J. Pharm. Res. Allied Sci., 2012, 1(4), 40-45.
21. Megala, K., Chenthilnathan, A., Babu, A.S. Validated RP-HPLC method for simultaneous determination of Telmisartan and Hydrochlorothiazide in pharmaceutical formulation. Int. J. Pharm. Res. Allied Sci., 2014, 3(3), 33-40.
22. Sunitha, N., Marihal, S.C., Sravanthi, J.S., Venu, A., Rao, C.V.N., Rao, B.A. Method development and validation of RP-HPLC method for the simultaneous estimation of Olmesartan and Cilnidipine in bulk and formulations. Int. J. Pharm. Res. Allied Sci., 2015, 4(3), 127-135.
23. Al-Bratty, M., Manoharan, G., Alhazmim, H.A. Development and validation of RP-HPLC method for the simultaneous estimation of Amlodipine and Nebivolol in raw and tablet formulation. Int. J. Pharm. Res. Allied Sci., 2016, 5(4), 37-44.
24. Nagamallika, G., Arunadevi, M. Rapid RP-HPLC method for the determination of Galantamine hydrobromide in pharmaceutical formulations. Indo Amer. J. Pharm. Res., 2013, 3(8), 6285-6290.
25. Obreshkova, D.P., Pangarova, T., Lazarova, T. HPLC determination of Galantamine and related alkaloids in Nivalin. Compt. Rend. Acad. Bulg. Sci., 2000, 53(9), 51-53.
26. Wu, J., Hua, Y., Tang, D., Gao, Y., Yin, X. Pharmacokinetics of Galantamine hydrobromide sustained-release tablets in beagle dogs. Chin. J. Pharmaceut., 2011, 6(1), 96-101.
27. Donnelly, R.F. Chemical stability of 4-aminopyridine capsules. Can. J. Hosp. Pharm., 2004, 57(5), 283-287.
28. Shrivastava, A., Gupta, V.B. Methods for the determination of limit of detection and limit of quantitation of the analytical methods. Chron. Young Sci., 2011, 2(1), 21-25.
29. International Conference on Harmonization of Technical Requirement for Registration of Pharmaceuticals for Human use, ICH harmonized tripartite Guideline, Validation of Analytical procedures Text and methodology Q2 (R1), 2005.
30. Ermer, J., Ploss, H.J. Validation in pharmaceutical analysis – Part II: central importance of precision to establish acceptance criteria and for verifying and improving the quality of analytical data. J. Pharm. Biomed. Anal., 2005, 37(5), 859-870.
31. Özdemir, E., Tatar, U,S. Highly sensitive HPLC method for the determination of Galantamine in human plasma and urine through derivatization with dansyl chloride using fluorescence detector. Luminescence, 2017, 32(7), 1145-1149.
32. Patel H.N., Patel A.V., Patel V.J., Dave J.B., Patel C.N. UV-spectrophotometric method development and validation for estimation of Galantamine hydrobromide in tablet dosage form. J. Chem. Pharm. Res., 2010, 2(2), 44-49.
33. Mittal, K., Kaushal, R., Mashru, R., Thakkar, A. Estimation of the Galanthamine using derivative spectrophotometry in bulk drug and formulation. J. Biomed. Sci. Eng., 2010, 3, 439-441.
34. Kus, S., Marczenko, Z., Obarski, N. Derivative UV-VIS spectrophotometry in analytical chemistry. Chern. Anal. Warsaw, 1996, 41(1), 899-927.