Original Article

Validation parameters of densitometric method for simultaneous determination of Galantamine hydrobromide and Pymadine

Dobrina Tsvetkova[1*], Stefka Ivanova[1]

[1]Medical University-Sofia, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, 2 Dunav Str., Sofia 1000, Bulgaria

Correspondence: Dobrina Tsvetkova, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Medical University-Sofia, Dunav str. N: 2, 1000, Sofia, Bulgaria. E mail: dobrinka30 @ mail.bg

ABSTRACT

The aim of the current investigation was the development and validation as per ICH requirements of the TLC-densitometric method with the detection at λ = 282 nm for the simultaneous determination of Galantamine hydrobromide and Pymadine in model mixtures. The aim was in accordance to the perspectives of multi-target therapy of Alzheimer's disease, and due to the fact that in the literature and pharmacopoeial articles the methods for simultaneous analysis of these components haven't been described. Selectivity was confirmed by the fact that in blank solution, Rf data weren't observed corresponding to Rf data of the active ingredients. The regression equations obtained demonstrated the linear relationship between the peak area and concentration: $y = 2.10^7.x + 25364$ (Galantamine hydrobromide) (LOD = $1.87.10^{-3}$ g/ml; LOQ = $6.22.10^{-3}$ g/ml); $y = 2.10^7.x + 65930$ (Pymadine) (LOD = $2.5.10^{-3}$ g/ml; LOQ = $8.35.10^{-3}$ g/ml). All the experimental data for the degree of recovery were included in the corresponding confidence interval: 93.54 % ÷ 97.32 % (Galantamine hydrobromide); 100.05 % ÷ 103.11 % (Pymadine). The results of precision suited the relevant intervals.

System suitability was confirmed by the lack of a statistically significant difference between the Rf values: 0.663 (Galantamine hydrobromide); 0.433 (Pymadine). The method was appropriate for the simultaneous determination of Galantamine hydrobromide and Pymadine in the model mixtures.

Keywords: Densitometry, Galantamine, Pymadine, validation, determination.

Introduction

Alzheimer is a neurodegenerative disease [1] associated with plaques and tangles in the brain [2]. The conventional therapy is with Galantamine (Fig. 1.), Donepezil and Rivastigmine [3]. A potent inhibitory activity against acetylcholinesterase possess the extracts of *Ocimum sanctum* [4] and *Cinnamon zeylanicum* [5].

In Alzheimer's disease [6], Galantamine improves the behavior [7] by blocking the reversible enzyme acetylcholinesterase and allosterically potentiation of α7-nicotinic acetylcholine receptors [8], and improves learning and memory alone and in combination with Pymadine [9].

Reactive oxygen species induce neurodegeneration in Alzheimer's disease [10], and Galantamine is appropriate for the treatmennt due to the antioxidant properties [11].

4-aminopyridine (Dalfampridine) (Fig. 1.), has been a drug for the symptomatic management of the multiple sclerosis [12] and it improves walking [13].

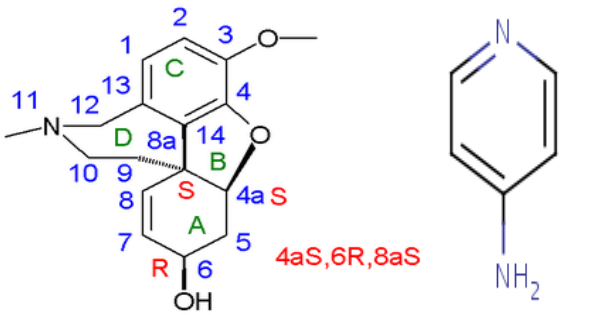

Figure 1. Chemical structures of Galantamine hydrobromide and 4-aminopyridine.

For the analysis of Galantamine in Nivalin, HPLC has been applied [14]. By High Performance Thin Layer Chromatography (HPTLC), Gallic acid, Curcumin and Quercetin are analysed [15]. TLC is often applied for the analysis of plant extracts: *Psidium guajava* [16], *Nelumbo nucif*era [17], Galantamine in

Access this article online	
Website:www.japer.in	**E-ISSN:** 2249-3379

How to cite this article: Dobrina Tsvetkova, Stefka Ivanova.Validation parameters of densitometric method for simultaneous determination of Galantamine hydrobromide and Pymadine. J Adv Pharm Edu Res 2018;8(4):9-14.

Source of Support: Nil, Conflict of Interest: None declared.

Galanthus species [18]. For Galantamine in *Galanthus species* extracts, Silicagel $G_{60}F_{254}$ or aluminium are used; spots are displayed by quenching the fluorescence at λ = 254 nm, or spraying with Dragendorf reagent or iodine vapour; and the mobile systems are: chloroform : ethylacetate : methanol = 20 : 20 : 10 v/v (*Galanthus nivalis L..;Galanthus elwesii Hook. Fil.*); chloroform : methanol : 25 % ammonia = 90 : 9 : 1 v/v (*Galanthus nivalis L.*) [18].

HPTLC has been applied for the analysis of the extracts of *Narcissus jonquilla Pipit* [19], and TLC-densitometry has been used for the quantification of Galantamine in the extracts of *Galanthus elwesii Hook* and *Galanthus nivalis L* [20].

TLC-extraction-spectrophotometric analysis has been developed for Galantamine in the extracts of *Galanthus nivalis L. subsp. cilicicus Baker* [21] after derivatization reaction with tropeolin 00 [18]. 3-hydroxy-4-aminopyridine has been analysed by TLC [22].

As a part of a new trend of multi-target drug therapy of Alzheimer's disease, future pharmacological investigations of combination Galantamine/Pymadine would be ongoing. On the other side, the quality control of the active substances has been important. In connection with these reasons, and due to the fact that in the literature and pharmacopoeial articles, the methods for the simultaneous analysis of Galantamine and Pymadine haven't been described, the aim of the current study was the development and validation of the TLC-densitometric method for the simultaneous identitification, and the determination of Galantamine hydrobromide and Pymadine in the mixture as per International Conference Harmonization [23] and validation requirements [24, 25].

Materials

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

II. Reagents with the pharmacopoeial purity: distilled water.

III. Solvents with the pharmacopoeial purity: methanol Chromasolv (99.9 %) (Sigma Aldrich, N: SZBD063AVUN1230); chloroform (Sigma Aldrich, N: SZBC186SVUN1888); acetone (Riiedel de Haën, N: STBG5763VUN1090); ethylacetate: (98.9 %) (Sigma Aldrich, N: 270989).

IV. TLC aluminium sheets Silicagel $G_{60}F_{254}$ (20 cm × 20 cm) (Merck, Germany, N:HX73683254).

Methods: TLC-densitometry.

I. Equipment.

TLC Densitometer TR 541a, wavelength scan mode λ = 282 nm, 10 µl micropipette (Hamilton, Bonaduz, Switzerland, N:18005701), TLC chamber (22 cm/12 cm/22 cm).

II. Chromatographic conditions.

Stationary phase: Silicagel $G_{60}F_{254}$, mobile phase: chloroform: acetone: ethylacetate: methanol = 20:10:5:5 v/v, UV detection at λ = 282 nm, start-front distance: 150 mm.

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

The accurate weighed quantities of the reference substances included: 0.01 g, 0.02 g, 0.03 g, 0.05 g, 0.1 g, 0.15 g, 0.3 g, (Galantamine hydrobromide) and 0.025 g, 0.05 g, 0.1 g, 0.15 g, 0.2 g, 0.25 g, 0.3 g, 0.35 g, 0.4 g (4-aminopyridine) were separatelly dissolved in the distilled water in 10.0 ml volumetric flasks for obtaining the solutions with concentrations of: 1.10^{-3} g/ml, 2.10^{-3} g/ml, 3.10^{-3} g/ml, 5.10^{-3} g/ml, 1.10^{-2} g/ml, $1.5.10^{-2}$ g/ml, 3.10^{-2} g/ml (Galantamine hydrobromide) and $2.5.10^{-3}$ g/ml, 5.10^{-3} g/ml, 1.10^{-2} g/ml, $1.5.10^{-2}$ g/ml, 2.10^{-2} g/ml, $2.5.10^{-2}$ g/ml, 3.10^{-2} g/ml, $3.5.10^{-2}$ g/ml, 4.10^{-2} g/ml (4-aminopyridine).

IV. The preparation of model mixtures of Galantamine hydrobromide and 4-aminopyridine for the validation of densitometric method with respect to the analytical parameters accuracy and internal precision (repeatability).

6 model mixes were prepared by measuring 0.2 g Galantamine hydrobrimide and 0.1 g 4-aminopyridine and dissolving in 10.0 ml distilled water.

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

The data were subjected to the linear regression analysis. From the regression equation of: y = a.x + b, the predictable absorbance value (Ap); the 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 [26].

Results and Discussion

I. Densitometric parameters for reference solutions of Galantamine hydrobromide and Pymadine.

The densitometric parameters for Galantamine hydrobromide (1.10^{-3} g/ml ÷ 3.10^{-2} g/ml) (Table 1.) and 4-aminopyridine (1.10^{-2} g/ml ÷ 4.10^{-2} g/ml) (Table 2.) were summarized.

Table 1. Densitometric parameters for reference standard Galantamine hydrobromide

Parameters	Reference standard Galantamine hydrobromide						
C [g/ml]	1.10^{-3}	2.10^{-3}	3.10^{-3}	5.10^{-3}	1.10^{-2}	$1.5.10^{-2}$	3.10^{-2}
Volume [µl]	10	10	10	10	10	10	10

Quantity [µg/spot]	10	20	30	50	100	150	300
R_f	0.66	0.66	0.67	0.66	0.67	0.66	0.66
Diameter (d)[cm]	0.1	0.2	0.3	0.6	1.2	1.3	1.6
Radius (r) [cm]	0.05	0.1	0.15	0.3	0.6	0.65	0.8
Area (S)[cm^2]	0.01	0.03	0.07	0.28	1.13	1.33	2.01
Spot area (A)	55200	83300	87600	115300	223600	336100	663000

Table 2. Densitometric parameters for reference standard 4-aminopyridine.

Parameters	Reference standard 4-aminopyridine						
C [g/ml]	1.10^{-2}	$1.5.10^{-2}$	2.10^{-2}	$2.5.10^{-2}$	3.10^{-2}	$3.5.10^{-2}$	4.10^{-2}
Volume [µl]	10	10	10	10	10	10	10
Quantity [µg/spot]	100	150	200	250	300	350	400
R_f	0.43	0.44	0.43	0.43	0.43	0.44	0.43
Diameter (d)[cm]	1.2	1.3	1.4	1.55	1.6	1.65	1.8
Radius (r) [cm]	0.6	0.65	0.7	0.78	0.8	0.83	0.9
Area (S) [cm^2]	1.13	1.33	1.54	1.91	2.01	2.16	2.54
Spot area (A)	232000	370400	419000	494200	576200	633100	703100

II. Validation of densitometric method for determination of Galantamine hydrobromide and Pymadine in model mixtures.

TLC method was validated for the analytical validation parameters' selectivity, linearity, limit of detection, limit of quantitation, accuracy (precision) and internal precision (repeatability) according toICH [23].

1. Selectivity

In the same manner like the reference solutions, a blank solution containing only a solvent was prepared and chromatographed. Selectivity was confirmed by the lack of R_f-data in the blank stolution corresponding to R_f-data of the active ingredients: Galantamine hydrobromide (R_f = 9.9/15 = 0.663); Pymadine (R_f = 6.5/15 = 0.433).

2. Linearity, LOD, LOQ.

The experimental results were subjected to the linear regression analysis. The calibration curves showed the linear relationship between the peak area A, and the concentration C which were illustrated in Fig. 2.

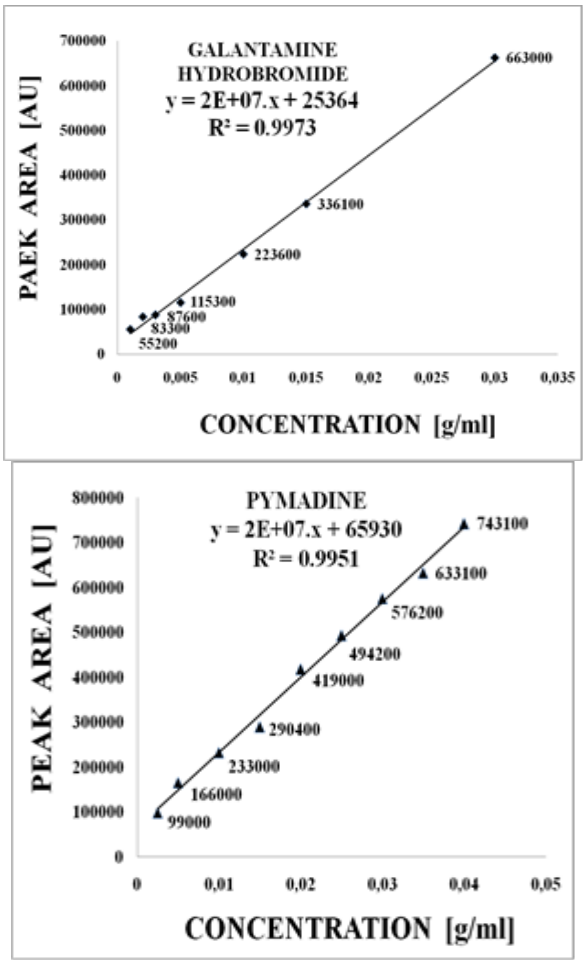

Figure 2. Linearity of Galantamine hydrobromide and Pymadine.

Linearity is the range within the signal from the detector that remains in the linear dependency from the concentration of the analyte [23].

For the analysis of linearity from the reference standards, a series of solutions with increasing concentrations of Galantamine hydrobromide (1.10^{-3} g/ml ÷ 3.10^{-2} g/ml) and 4-aminopyridine (1.10^{-2} g/ml ÷ 4.10^{-2} g/ml) were prepared. The peak areas were measured, and the results were included in Table 1. and 2.

In Table 3., the parameters of the regression equations, which demonstrated the linear relationship between the peak area and the concentration at the corresponding concentration intervals were described. Linearity was characterized by coefficients of the linear regression: $R^2 > 0.995$.

Table 3. Parameters of regression equations for Galantamine hydrobromide and Pymadine

N:	Parameter	Galantamine hydrobromide	Pymadine
1.	Linear interval [g/ml]	$1.10^{-3} \div 3.10^{-2}$	$2.5.10^{-3} \div 4.10^{-2}$
2.	Regression equation	$y = 2.10^7.x + 25364$	$y = 2.10^7.x + 65930$
3.	Slope (a)	2.10^7	2.10^7
4.	Standard slope error	491256	445422
5.	Inrersept (b)	25364	65930
6.	Standard inrersept error	6601	10610
7.	Correlation coefficient (R^2)	0.9973	0.9951

The results of the analysis of the standard solutions with increasing concentration of Galantamine hydrobromide (Table 4.) and 4-aminopyridine (Table 5.) for the investigation of linearity, LOD and LOQ were summarized as follows:

C [g/ml] – concentration, A – measured peak area, Ap – calculated by the calibration curve peak area.

LOD and LOQ were based on the regression equations: $y = 2.10^7.x + 25364$ (Galantamine hydrobromide) and $y = 2.10^7.x + 65930$ (Pymadine) (Table 4.) by the application of RMSE-method [23].

Table 4. Linearity, LOD and LOQ for Galantamine hydrobromide

N:	C [g/ml]	A	A_p	$E = \lvert A_p\text{-}A \rvert$	$E^2 = [\lvert A_p - A \rvert]^2$
1.	1.10^{-3}	55200	46372	8828	77933584
2.	2.10^{-3}	83300	67381	15919	253414561
3.	3.10^{-3}	87600	88389	789	622521
4.	5.10^{-3}	115300	130406	15106	228191236
5.	1.10^{-2}	223600	235448	11848	140375104
6.	$1.5.10^{-2}$	336100	340490	4390	19272100
7.	3.10^{-2}	663000	655615	7385	54538225

$\sum E2 = 774347331$ $E1 = \frac{\sum E2}{n-2} = 154869466$ $RMSE = \sqrt{154869466} = 12444.66$

LOD= $(3.12444.66)/2.10^7 = 1.87.10^{-3}$ g/ml LOQ = $(10.12444.66)/2.10^7 = 6.22.10^{-3}$ g/ml

Table 5. Linearity, LOD and LOQ for Pymadine.

N:	C [g/ml]	A	A_p	$E = \lvert A_p - A \rvert$	$E^2 = [\lvert A_p - A \rvert]^2$
1.	$2.5.10^{-3}$	99000	107856	8856	78428736
2.	5.10^{-3}	166000	149783	16217	262991089
3.	1.10^{-2}	233000	233636	636	404496
4.	$1.5.10^{-2}$	290400	317489	27089	733813921
5.	2.10^{-2}	419000	401342	17658	311804964
6.	$2.5.10^{-2}$	494200	485195	9005	81090025
7.	3.10^{-2}	576200	569047	7153	51165409
8.	$3.5.10^{-2}$	633100	652900	19800	392040000
9.	4.10^{-2}	743100	736753	6347	40284409

$\sum E2 = 1952023049$ $E1 = \frac{\sum E2}{n-2} = 278860436$ $RMSE = \sqrt{278860436} = 16699.11$

LOD= $(3.16699.11)/2.10^7 = 2.5.10^{-3}$ g/ml LOQ= $(10.16699.11)/2.10^7 = 8.35.10^{-3}$ g/ml

III. Accuracy and internal precision (repeatability).

6 model mixtures containing 20 mg Galantamine hydrobromide (G) and 10 mg Pymadine (P) were analyzed by TLC, and the results for: peak area (A): AG_{20}, AP_{10} and Chauvenet's criterion for peak area (UA): UA_{G20}, UA_{P10} were summarized in Table 6.

Table 6. Peak area and Chauvenet's criterion for Galantamine hydrobromide and Pymidine in mixtures.

N:	A_{P10}	UA_{P10}	A_{G20}	UA_{G20}
1.	266200	1.56	414500	0.04
2.	267500	0.86	417900	0.93
3.	269500	0.20	408700	1.69
4.	270000	0.47	416300	0.47
5.	270500	0.74	417700	0.87
6.	271000	1.01	412700	0.55
$\bar{X}$±SD	269117±1871		414633±3516	
RSD [%]	0.7		0.85	

The contents of Galantamine hydrobromide [G_{20}] and Pymadine [P_{10}] were calculated by the application of calibration curve method, using the peak area of the components. The results for accuracy and repeatability were summarized in Table 7.

Table 7. Accuracy and repeatability for model mixtures of Galantamine hydrobromidePymadine.

N:	$[P_{10}]$ [mg/ml]	$R[P_{10}]$ [%]	$U[P_{10}]$	$[G_{20}]$ [mg/ml]	$R[G_{20}]$ [%]	$U[G_{20}]$
1.	10.01	100.1	1.67	19.46	96.34	0.06
2.	10.08	100.8	0.89	19.63	97.18	0.89
3.	10.18	101.8	0.22	19.17	94.90	1.67
4.	10.20	102.0	0.44	19.55	94.90	0.44
5.	10.23	102.3	0.78	19.62	95.24	0.83
6.	10.25	102.5	1.0	19.37	94.03	0.56
$\bar{X}$±SD	10.16± 0.09			19.47± 0.18		
$\bar{R}$[%]±RSD [%]		101.58± 0.93			95.43± 1.19	
SD	0.09	0.94		0.18	1.14	
RSD [%]	0.89	0.93		0.92	1.19	
$S\bar{X}$	0.04	0.38		0.07	0.47	
P [%]	99.0	99.0		99.9	99.0	
t	4.03	4.03		6.86	4.03	
$t.S\bar{X}$	0.16	1.53		0.48	1.89	
$\bar{X}+ t.S\bar{X}$÷ $\bar{X}- t.S\bar{X}$	10.0÷ 10.32	100.05÷ 103.11		18.99÷ 19.95	93.54÷ 97.32	
E [%]	0.39	0.37		0.36	0.49	

In Table 7., the results for: N – number of measurements (1÷6); $[G_{20}]$, $[P_{10}]$ – quantity of components; $R[G_{20}]$, $R[P_{10}]$ – degree of recovery [%]; $U[G_{20}]$, $U[P_{10}]$ – Chauvenet's criterion; $\bar{X}$– arithmetic mean; SD – standard deviation; RSD [%] –related standard deviation; $S\bar{X}$– mean square error; P – confidence probability [%]; t – coefficient of Student; $\bar{X} \pm t.S\bar{X}$– confidence interval; E – relative error [%], were summarized.

Accuracy is the degree of the correspondence between the obtained average result of the repeated analysis and the actual values. For the model mixtures, accuracy was represented by the degree of recovery R [%] ± RSD [%] as per ICH guidelines [23]: 95.43 % ± 1.19 % (Galantamine hydrobromide); 101.58 % ± 0.93 % (Pymadine). SD and RSD were lower than 1.5, which proved the correspondence between the obtained results and the actual values.

Repeatability is characterized by the uncertainty of the results, which includes SD, RSD and the confidence interval [23], and in this study, it was investigated on Galantamine hydrobromide and Pymadine content in 6 model mixtures. At the confidence level of P = 99.0 %, all the data for amounts suited the corresponding confidence intervals: 18.99 mg ÷ 19.95 mg (Galantamine hydrobromide);10.0 mg ÷ 10.32 mg (Pymadine). The data for Chauvenet's criterion were lower than the maximum permissible value (U = 1.73; N = 6), which confirmed that no statistically significant difference between the obtained quantities was observed, and this proved the repeatability of the results.

IV. System suitality test

The system suitability was confirmed by the lack of a statistically significant difference between the values for R_f: $\bar{X}_{Rf}$ = 0.663 (Galantamine hydrobromide); $\overline{X}_{Rf}$ = 0.433 (Pymadine) (Table 8.).

Table 8. Rf for Galantamine hydrobromide and Pymadine in model mixtures.

N:	Galantamine hydrobromide R_f	Pymadine R_f
1.	0.66	0.43
2.	0.67	0.44
3.	0.66	0.43
4.	0.66	0.43
5.	0.67	0.44
6.	0.66	0.43
$\bar{X}$±SD	0.663÷0.005	0.433÷0.005
RSD [%]	0.75	1.15

Conclusion

Regression equations demonstrated a linear relationship between the peak areas and concentrations. All the experimental data for the degree of recovery and for the repeatability were included in the corresponding confidence interval. The system suitability was confirmed. The developed and validated TLC-densitometric method was appropriate for the simultaneous determination of Galantamine hydrobromide and Pymadine.

Conflicts of Interests

All authors had none to declare.

References

1. Nikolova I., Danchev N., Lamvov N., Grudeva V. Neurodegenerative diseases in geriatry – opportunities of current pharmacotherapy. Clin. Homeopathy 2010; 1(1): 3-6.
2. Ballard C., Gauthier S., Corbett A., Brayne C., Aarsland D., Jones E. Alzheimer's disease. Lancet 2011; 377(9770): 1019-1131.
3. Danchev N., Nikolova, I. Pharmacological treatment of cognitive impairements in Alzheimer's disease. Autonomic Autocoid Pharmacol. 2006; 26(1): 46-49.
4. Kandhan T.S., Thangavelu L., Roy A. Acetylcholinesterase activity of *Ocimum sanctum* leaf extract. J. Adv. Pharm. Edu. Res. 2018; 8(1): 41-44.
5. Jain P., Thangavelu L, Roy A. Acetylcholinesterase activity of *Cinnamon zeylanicum* extract. J. Adv. Pharm. Edu. Res. 2017; 7(4): 482-448.
6. Gaudig M., Richarz U., Han J., Van Baelen B., Schäuble B. Effects of Galantamine in Alzheimer's disease: double-blind withdrawal studies evaluating sustained versus interrupted treatment. Curr. Alzheim. Res. 2011; 8(7): 771-780.
7. Marco L., Carreiras M.C. Galanthamine, a natural product for the treatment of Alzheimer's disease. Recent. Pat. CNS Drug. Discov. 2006; 1(1): 105-111.
8. Takada-Takatori Y., Kume T., Izumi Y., Ohgi Y., Niidome T., Fujii T., Sugimoto H., Akaike A. Roles of nicotinic receptors in acetylcholinesterase inhibitor-induced neuroprotection and nicotinic receptor up-regulation. Biol. Pharm. Bull. 2009; 32(3): 318-324.
9. 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.
10. Obreshkova D. Reactive oxygen species induced neurodegeneration in Alzheimer's disease. Pharmacia 2013; 60(1): 71-82.
11. Tsvetkova D., Obreshkova D., Zheleva-Dimitrova D., Saso L. Antioxidant activity of Galanthamine and some of its derivatives. Curr. Med. Chem. 2013; 20(36): 4595-4608.
12. Espejo C., Montalban X. Dalfampridine in multiple sclerosis: from symptomatic treatment to immunomodulation. Clin. Immunol. 2012; 142(1): 84-92.
13. 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.
14. Obreshkova D. Determination of Galanthamine and related alkaloids in Nivalin. Compt. Rend. Acad. Bulg. Sci. 2000; 53(9): 51-54.
15. Thakker V.Y., Shah V.N., Shah U.D., Suthar M.P. Simultaneous estimation of Gallic acid, Curcumin and Quercetin by HPTLC method. mice. J. Adv. Pharm. Edu. Res. 2011; 1(1): 70-80.
16. Sarkar J., Pal S., Bhattacharya S., Biswas M. Thin layer chromatographic profiling and evaluation of analgesic activity of Psidium guajava leaf extracts in mice. J. Adv. Pharm. Edu. Res. 2011; 1(3): 177-183.
17. Bera S., Bhattacharya S., Pandey J.N., Biswas M. Thin layer chromatographic profiling and evaluation of analgesic activity of Nelumbo nucifera leaf extracts in Swiss Mice J. Adv. Pharm. Edu. Res. 2011; 1(6): 259-265.
18. Novikova I.Y., Tulaganov A.A. Structure of chemical compounds, methods of analysis and process control.

Physicochemical methods for the analysis of Galanthamine. Pharm. Chem. J. 2002; 36(11): 623-627.
19. Mroczek T., Mazurek J. Pressurized liquid extraction and anticholinesterase activity-based thin-layer chromatography with bioautography of Amaryllidaceae alkaloids. Anal. Chim. Acta 2009; 633(2): 188-196.
20. Sidjimova B., Berkov S., Popov S., Evstatieva L. Galanthamine distribution in Bulgarian Galanthus spp. Pharmazie 2003; 58(12): 935-936.
21. Kaya G.I., Gözler B. Quantitative and cytotoxic activity determinations on Galanthus nivalis subsp. cilicicus. Fitoter. 2005; 76(3-4): 340-343.
22. Caggiano A., Blight A. Identification of metabolites of Dalfampridine (4-aminopyridine) in human subjects and reaction phenotyping of relevant cytochrome P450 pathways. J. Drug. Assess. 2013; 2(1): 117-126.
23. International Conference on Harmonization of Technical Requirement for Registration ofPharmaceuticals for Human use, ICH harmonized tripartite Guideline, Validation of Analytical procedures Text and methodology Q2 (R1), 2005.
24. Gamil A.M. Validation as applied for pharmaceutical processes. J. Adv. Pharm. Edu. Res. 2015; 5(2): 77-86.
25. Sudha T., Krishana K.V, Nukala P.C.S., Mishal, Saloman R.T., Ganesan V. Method development and validation – a review. J. Adv. Pharm. Edu. Res. 2013; 2(3): 146-176.
26. 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.