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VALIDATED SPECTROFLUORIMETRIC DETERMINATION OF DALFAMPRIDINE IN ITS SYNTHETIC MIXTURE AND SPIKED HUMAN PLASMA THROUGH DERIVATIZATION WITH FLUORESCAMINE

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Abstract- A sensitive, simple and selective spectrofluorimetric method was developed for the determination of dalfampridine. The method was based on the reaction between the drug and fluorescamine in borate buffer of pH 8.5 to give highly fluorescent derivative that was measured at 485 nm using an excitation wavelength of 385 nm. The optimum reaction conditions were determined by factorial design of experiment and the method was applied for the determination of dalfampridine over the concentration range of 20-100ng.mL^{-1}. The suggested method was applied successfully for a synthetic mixture simulated to its tablet dosage form. The mean recovery from synthetic mixture was found to be 98.89%±1.17 with no interference from excipients. Furthermore, the method was applied for the determination of dalfampridine in spiked human plasma, the mean % recovery was 96.74±1.86.

Keywords- dalfampridine, fluorescamine, spectrofluorimetry, factorial design

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Introduction

Dalfampridine is the first drug approved in the United States by FDA to improve walking in patients with multiple sclerosis. It is chemically known as 4-aminopyridine or fampridine. Ampyra® is an extended release tablet formulation of dalfampridine which was previously called Fampridine-SR. Fampridine is a potassium channel-blocker that enhances conduction in focally demyelinated axons, improves synaptic transmission and potentiates muscle contraction. It has shown efficacy in patients with all five major types of multiple sclerosis namely relapsing, remitting, secondary progressive, progressive relapsing and primary progressive [1-4]. Literature survey revealed that few analytical method has been reported for determination of 4-AP including HPLC method [5] and gas chromatographic method [6].

Experimental

Apparatus

fluorescence measurements were carried out on a FP-6300 spectrofluorimeter (Jasco, Japan) equipped with a 150 W xenon lamp and 1 cm quartz cells. The slit widths of both the excitation and emission monochromators were set at 5 nm. The calibration and linearity of the instrument were frequently checked with standard quinine sulphate. pH meter Model 211 a product of HANNA Instruments Inc. Centrifuge (Hettich, Germany).

Reagents and Solution

- Fluorescamine and dalfampridine were purchased from Sigma Aldrich.
- Fluorescamine 1.0 mg.mL^{-1} was prepared in acetonitrile. The solution is stable for at least 7 days if kept in the refrigerator.
- Borate buffer solution pH 8.5 was prepared by dissolving 618.9 mg of boric acid and 745.6 mg of potassium chloride in 200 mL distilled water and adjusting pH by 0.2 M NaOH.
- Human plasma was kindly supplied from Tanta Blood Bank. Egypt.
- Drug stock solution was prepared by dissolving 25 mg 4-AP in 25 mL of distilled water to obtain a solution with concentration 1mg mL^{-1}, then aliquot of this solution is diluted to obtain working solution of a concentration of 10.0 µg. mL^{-1}.
- All the reagents used were of analytical grade.

Derivatization Procedure

An aliquot from the working solution of the drug was transferred into 5.0 mL standard flask then 250 µL of fluorescamine solution (1.0mg.mL^{-1}) was added, followed by 0.5 mL of borate buffer pH 8.5, the content mixed well and volume was adjusted to 5.0 mL with water. The fluorescence intensity of the resulting solution was measured after 5 min at room temperature at 485 nm after excitation at 385 nm. The observed fluorescence was corrected by subtracting the fluorescence intensity measured using a reagent blank prepared in the same manner using water instead of the drug.

Construction of Calibration Curve

Different aliquots of the working standard solution of 4-AP in water (10.0 µg.mL^{-1}) were quantitatively transferred into a series of 5.0 ml

standard flasks so as to obtain the drug within the concentration range of 20-100 ng.mL^{-1}and derivatization procedure was performed as mentioned above. The % relative fluorescence (R.F.) was plotted versus the final concentration to get the calibration graph Regression equation was computed.

Assay of Synthetic Mixture of Drug and Excipients

A synthetic mixture of drug with excipients simulated to its dosage form Ampyra® because it is not available on the local market, was prepared by mixing 10 mg of dalfampridine with 86 mg of microcrystalline cellulose, 2 mg of silicon dioxide and 2 mg of magnesium stearate. The mixture was transferred into 100 mL volumetric flask then dissolved in 50 mL water and sonicated for 10 minutes. The volume was brought to 100 mL with water and final solution was filtered then aliquot of the filtrate was further diluted with water to get a final concentration of 10.0 µg.mL^{-1}. Different aliquots were taken from this solution to prepare three concentrations covering the linearity range (20, 40, 80) ng.mL^{-1}. Derivatization procedure was carried out and the % recovery was calculated from the regression equation.

Assay of the Drug in Spiked Human Plasma

Aliquots of 0.5 mL of human plasma were spiked with different concentration levels of 4-AP, transferred into centrifugation tubes, then 2.5mLof acetonitrile was added to each and centrifuged at 3000 rpm for 15 min. One mL of clear supernatant was taken and fluorogenic reaction was carried out. The fluorescence intensity of the solution was measured at 485 nm with excitation at 385 nm. A blank experiment was carried out simultaneously. The nominal content of the drug in plasma was determined by using the corresponding regression equation.

Results and Discussion

Only HPLC and GC methods were reported for determination of 4-AP and no spectroscopic method was developed for its determination. Fluorimetry is considered one of the most convenient analytical techniques, because of its inherent simplicity, high sensitivity, low cost, and wide availability in most quality control laboratories. No attempt has yet been made for the fluorimetric determination of 4-AP. 4-amino pyridine is characterized by a very weak fluorescence in a variety of solvents [7] hence its derivatization with fluorogenic reagent was necessary for its fluorimetric determination. Fluorescamine was chosen as a derivatizing reagent because it forms highly fluorescent derivatives with primary amines under relatively mild reaction conditions [8]. It was found that 4-AP reacts with fluorescamine and forms a highly fluorescent product that exhibited maximum fluorescence intensity at (λem) 485 nm after excitation at wavelength (λex) of 385 nm. The excitation and emission spectra for the reaction product of 4-AP with fluorescamine are given in [Fig -1]. Factorial design of experiment was made to study the reaction condition between dalfampridine and fluorescamine. One-factor-at-a-time method (OFAT) is inefficient, can give misleading results, and in general it should be avoided as we cannot be sure that the influence of a given factor will be the same whatever the levels of the other factors while factorial design gives the researcher the ability to study interactions between factors and provides a greater chance of finding optimum conditions by varying all factors together (factorial design) preferred [9].

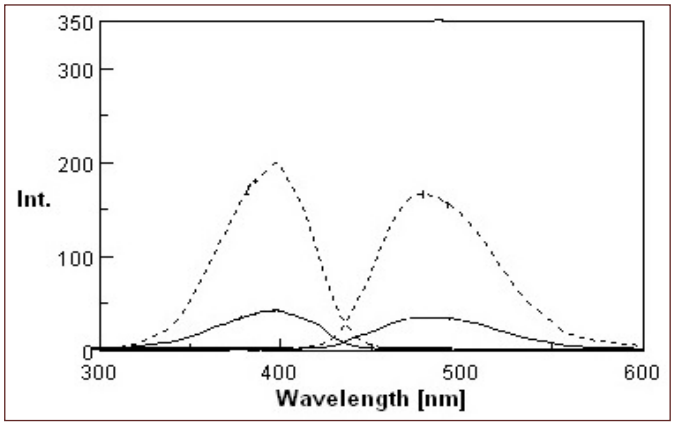


Fig. 1- The excitation and emission spectrum of the reagent blank (—) and the reaction product (----)

Optimization of the Reaction Conditions

Factors affecting the reaction of 4-AP with fluorescamine including pH and the concentration of the reagent were studied by varying each in turn while keeping the others constant (one factor at a time) to determine the upper and lower level. The effect of pH was studied over the range (8-10.5) using borate buffer and the effect of concentration of the reagent was studied by using different volume (100-500 µL) of fluorescamine (1.0 mg. mL^{-1}). It was found that the reaction revealed very low fluorescence intensity below 100 mL of fluorescamine while the fluorescence intensity increased by increasing the volume but the fluorescence intensity of the blank also increased so 100 µL and 250 µL were selected as lower and upper level respectively while pH 8.5 was taken as lower level and pH 9 as upper level then 2 ^{2} factorial design was applied to optimize the reaction condition. The result of factorial design was shown in [Table-1] and [Table-2].

Table 1- Results of factorial design experiments

No. of run	Volume of Fluorescamine (µL)	pH	Fluoresence intensity
1	100	8.5	250.66
2	100	9	507.13
3	250	8.5	700.45
4	250	9	617.11

Table 2- Main effect of factors and interaction effect

Factor	Effect
Fluorescamine volume (µL)	279.89
pH	86.56
Fluorescamine *pH	-169.91

It was found that the effect of fluorescamine volume was greater than pH effect and there was negative interaction between them. So maximum fluorescence intensity was given by adding 250 µL of fluorescamine in presence of borate buffer pH 8.5.

Stoichiometry of the Reaction

The stoichiometry of the reaction was studied using the limiting logarithmic method [10]. Two straight lines were obtained upon using increasing concentrations of the drug while keeping the concentration of the reagent constant [Fig-2A] and upon using increasing concentrations of the reagent while keeping the concentration of the drug constant [Fig-2B]. The two lines gave two slopes with the values of 1.151 and 1.023 respectively, therefore the stochiometry is 1:1. Hence the reaction is proposed to proceed as shown in [Scheme-1].

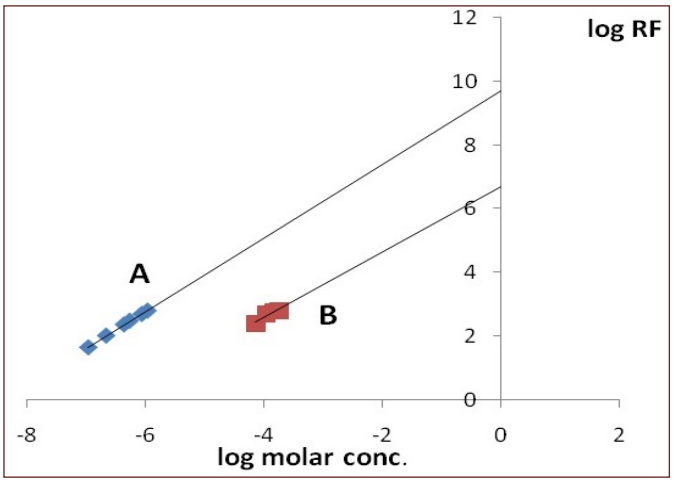
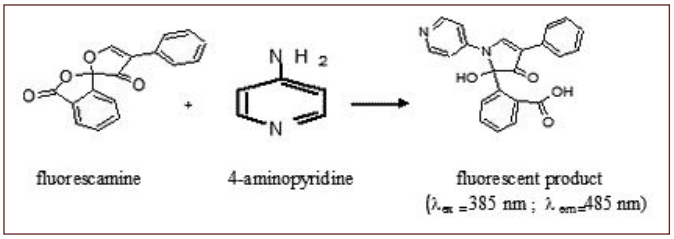


Fig. 2- Limiting logarithmic plots for the molar reactivity of (4-AP) with fluorescamine. (A) log R.F. versus log [4-AP] with [fluorescamine] kept at 1.79×10^{-4} M; (B) log R.F. versus log [fluorescamine] with [4-AP] kept at 1.06×10^{-6} M.



Scheme 1- The suggested pathway for derivatization of 4-AP with fluorescamine

Validation of the Method

The proposed method was validated regarding linearity, specificity, accuracy, repeatability and intermediate precision according to ICH Q2 (R1) recommendations[11].

Linearity

The calibration graph obtained by plotting the values of the fluorescence intensity versus the final concentration of the drug was found to be rectilinear over the concentration ranges 20-100 ng. mL^{-1}. The linear regression equation was derived by least-squares treatment of the calibration data. [Table-3] summarizes the performance data and statistical parameters for the proposed method, including concentration range, linear regression equation, correlation coefficient, standard deviation of the intercept (S_a) and slope (S_b), and SD of residuals (S_{xy}). The high value of the correlation coefficient (>0.999) indicates good linearity over the working concentration range.

Table 3- Performance data and statistical parameters for determination of 4-AP by the proposed method

Parameters	Results
Concentration range ($ng.mL^{-1}$)	20-100
Limit of detection (LOD) $ng.mL^{-1}$	0.6
Limit of quantitation (LOQ) $ng.mL^{-1}$	1.7
Regression Parameters	
Slope ± SD (S_b)	6.597±0.0499
Intercept ± SD (S_a)	-24.54±2.95
SD of residual (S_{xy})	3.865
Correlation coefficient (r)	0.9998

(S_b) standard deviation of the slope; (S_a) standard deviation of the intercept; (S_{xy}) standard deviation of the residuals

Limit of Quantitation and Limit of Detection

LOQ and LOD were calculated according to the following equations [11]:

$$LOQ = 10\ \sigma/S$$

$$LOD = 3.3\ \sigma/S$$

where σ is the standard deviation of blank readings, and S is the slope of the calibration curve

LOD and LOQ were also calculated practically and they were found to be 10 $ng.mL^{-1}$ and 20 $ng.mL^{-1}$ respectively.

Accuracy

To test the validity of the proposed method It was applied to the determination of pure sample of 4-AP over the chosen concentration range(30, 60, 90) $ng.mL^{-1}$. The accuracy was determined by calculating mean % recovery ± SD, as shown in [Table-4].

Table 4- Results of recovery studies of 4-AP in pure form

Conc. Taken ($ng.mL^{-1}$)	Conc. found ($ng.mL^{-1}$)	% Recovery	Mean %recovery *±SD
30	30.7	102.34	
60	60.72	101.19	101.22±1.1
90	90.12	100.14	

**Average of three determinations*

Precision

Repeatability (intra-day) was performed over a specified concentration range through replicate analysis of three concentrations of 4-AP in pure form on three successive occasions. The results are presented in [Table-5]. Intermediate precision (inter-day) was tested by repeated analysis of 4-AP in pure form using selected concentrations for a period of 3 successive days. High % recovery, low SD and low % RSD indicate high accuracy and precision of the proposed method, respectively.

Table 5- Results of precision study for determination of 4-AP by the proposed method

Conc, taken ($ng.mL^{-1}$)	Intra-day		Inter-day	
	Mean % recovery* ±SD	RSD%	Mean % recovery* ±SD	RSD%
20	100.2±1.9	1.9	102±1.16	1.13
50	100.2±0.8	0.79	100.5±1.14	1.14
80	98.82±0.7	0.7	100.03±0.47	0.47

**Average of three determinations*

Specificity and Interference

The % recovery of the drug in the synthetic mixture show no interference from excipients listed by the manufacturer such as microcrystalline cellulose, silicon dioxide and magnesium stearate so that the method is specific. The results are summarized in [Table-6].

Table 6- Statistical analysis of the results obtained by the proposed method for the determination of 4-AP in the synthetic mixture.

Conc. taken ($ng.mL^{-1}$)	Conc. found ($ng.mL^{-1}$)	% Recovery	Mean % recovery *±SD	RSD%
20	19.63	98.15		
40	40.1	100.25	98.89±1.17	1.18
80	78.6	98.25		

**Average of three determinations*

Robustness

The robustness of the procedure adopted in the proposed method was demonstrated by the constancy of the fluorescence intensity with the deliberated minor changes in the experimental parameters. Changes included the volume of the reagent (250±50 µL), the pH (8.5±0.2).

These minor changes that may take place during the experimental operation did not affect the fluorescence intensity of the reaction product.

Application to Spiked Human Plasma

The high sensitivity of the proposed method allowed the determination of 4-AP in spiked human plasma. Dalfampridine is mostly unbound to plasma protein (97-99%) and average peak plasma concentrations following oral dosing of 10 mg and 20 mg were 25 $ng.mL^{-1}$ and 49 ng/mL^{-1}, respectively, 3 to 4 hrs. post administration which is within the working concentration range of the proposed method [4]. The sample preparation only involves protein precipitation using acetonitrile. Liquid-liquid extraction using dichloromethane was tried but it was unable to totally extract the drug from plasma. Hydroxylic solvents such as methanol have been found to react with fluorescamine to form addition products and should be avoided as they drastically reduce the reactivity of fluorescamine toward primary amines [12-14], so acetonitrile was used for plasma protein precipitation. The method was simple and no evaporation step was required [15]. The validity of the method was proved for analysis of the drug in spiked human plasma according to ICH Q (linearity, accuracy, intraday precision, interday precision, specificity, LOD and LOQ). The results are shown in [Table-7].

Table 7- Validation of the proposed method for determination of dalfampridine in spiked human plasma

Parameter	Results
Concentration range ($ng.mL^{-1}$)	20-100
Limit of detection (LOD) ($ng.mL^{-1}$)	0.72
Limit of quantitation (LOQ) ($ng.mL^{-1}$)	2.37
Regression Parameters	
Slope ± SD (S_b)	3.329±0.1649
Intercept ± SD (S_a)	-18±10.537
SD of residual (S_{xy})	10.537
Correlation coefficient (r)	0.9903
Accuracy (mean*±RSD, %)	96.74±1.86
Precision	
Intraday (mean*±RSD, %)	98.57±1.42
Interday (mean*±RSD, %)	97.67±1.07

**Average of three determinations; (S_b) standard deviation of the slope; (S_a) standard deviation of the intercept; (S_{xy}) standard deviation of the residuals*

Stability

The stability of final measured sample solutions was examined and responses were found to be stable for 7 days at room temperature.

Conclusion

The proposed method is simple, accurate, sensitive and less tedious than chromatographic procedures. Also, it is suitable for the determination of 4-AP in the drug substance and spiked human plasma. The proposed method can be recommended for routine quality control analysis of dalfampridine where sophisticated equipments are unavailable.

Conflict of Interest: None declared.

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