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DEVELOPMENT OF AN HPLC METHOD FOR THE DETERMINATION OF FAMPRIDINE (DALFAMPRIDINE) IN HUMAN PLASMA BY FLUORIMETRIC DERIVATIZATION AND APPLICATION TO A PROTOTYPE PHARMACOKINETIC STUDY

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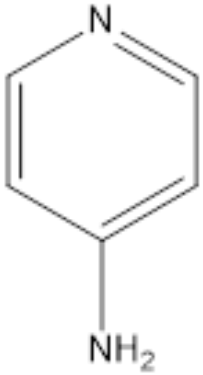
Fampridine (dalfampridine) is used to improve walking in people who have multiple sclerosis. In this study, a new, fast and sensitive HPLC method with fluorometric detection was developed for the determination of fampridine in human plasma and applied to a pharmacokinetic study. Fampridine was precolumn derivatized with 7-chloro-4-nitrobenzofurazan (NBD-Cl) and the fluorescent derivative was separated on a C18 (150 × 4.6 mm × 2.6 µm) analytical column at 30 ºC using a mobile phase composed of acetonitrile – 0.1% o-phosphoric acid in water (80:20, v/v) by isocratic elution with flow rate of 1.0 mL min^{-1}. The method was based on the measurement of the derivative using fluorescence detection (λ_{ex}= 310 nm, λ_{em} = 365 nm). The retention time of fampridine is 4.10 ± 0.02 min. This currently developed method was validated according to EMA criteria by evaluating the specificity, linearity, precision, accuracy and robustness. The method was determined to be linear in a concentration range of 0.01–20 ng mL^{-1} with the correlation coefficient of 0.9996. LOD and LOQ were found to be 0.003 and 0.01 ng mL^{-1}, respectively. Intraday and interday RSD values were less than 2.45%. The plasma concentration-time profile and pharmacokinetic parameters such as AUC_{0-t}, $AUC_{0-\infty}$, C_{max}, t_{max}, $t_{1/2}$, were calculated according to the assays. The presented method can certainly be used for bioequivalence and bioavailability investigations and routine analysis of the drug in plasma.



INTRODUCTION

The first medication specifically approved by the FDA in 2010 to improve walking in people with multiple sclerosis (MS), a disease in which the nerves do not function properly and can cause weakness, numbness, loss of muscle coordination, and problems with vision, speech, and bladder control (Fig. 1), is fampridine (dalfampridine) extended release tablets (dalfampridine-ER, also known as prolonged-modified or sustained-release fampridine tablets in some countries).[1,2] Two Phase 3 clinical trials that showed a substantial increase in walking speed compared to a placebo among patients who responded to therapy served as the basis for this approval.[3,4] Fampridine can be taken either by alone or in combination with other drugs that manage MS symptoms. Fampridine belongs to a group of drugs known as potassium channel blockers.[5] It functions by enhancing the impulses that the brain sends through MS-damaged neurons.[6]

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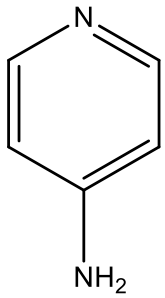


Fig. 1 – Structure of fampridine (dalfampridine).

Previously known as Fampridine-SR, Ampyra® is a dalfampridine extended release tablet formulation. Relapsing, remitting, secondary progressive, progressive relapsing, and primary progressive are the five main forms of multiple sclerosis in which it is effective in individuals.[7-10] Stated differently, dalfampridine functions to ensure that the available current is sufficiently high to promote conduction in exposed demyelinated axons in MS patients. Additionally, it relieves conduction blockages in demyelinated axons, which promotes neuromuscular and synaptic transmission.[11]

Fampridine (dalfampridine) can be determined using a limited number of analytical techniques, including high-performance liquid chromatography,[12,13,14] high-performance liquid chromatography-tandem mass spectrometry,[15,16,17] and gas chromatography-tandem mass spectrometry.[18] Additionally, only HPLC offers fampridine stability methods.[19] All these studies were performed in pharmaceutical formulations and rat plasma. The derivatization of fampridine with the NBD-Cl molecule for biological matrices has not yet been disclosed. Despite not being a fluorogenic molucule, fampridine is a molecule that can undergo fluorogenic derivatization processes because of the primary amine groups in its structure. The fluorogenic reagent 7-chloro-4-nitrobenzofurazan (NBD-Cl), which is commonly preferred due to its straightforward reaction technique, quick reactivity, and excellent sensitivity, was used in the pre-column derivatization process. For the purpose of determining amines, amino acids, thiol, and sulphyryl groups, it was employed as a fluorogenic agent.[20,21] When the reagent is hydrolyzed in alkali media, it can also be used to identify compounds that contain carbamate.[22] The purpose of this project is to create and validate an HPLC-FL method for detecting fampridine in human plasma and then use the new technique in a pharmacokinetic prototype study. Following fampridine injection, the plasma samples of the healthy volunteer were used for pharmacokinetic analysis with the ethics committee's approval.

EXPERIMENTAL

Chemicals and Reagents

Shanghai Yingxuan Pharmaceutical Science & Technology (China) provided the fampridine, while the local pharmacy sold Ampyra® extended release pills with 10 mg of fampridine. The following were provided by Merck (Darmstadt, Germany): acetonitrile (HPLC grade), orthophosphoric acid (HPLC grade), monobasic dihydrogen phosphate and dibasic monohydrogen phosphate, hydrochloric acid (analytical grade), methanol (analytical grade), and chloroform (analytical grade). The source of NBD-Cl was Sigma Aldrich, located in St. Louis, USA. A Human (Japan) ultrawater filtration system was used to purify the water.

Solutions

Standard solutions of 0.01 to 20 ng mL^{-1} were created by diluting a stock solution of fampridine (0.1 mg mL^{-1}) in methanol. In 50 milliliters of water, 2.0209 grams of sodium phosphate dibasic and 0.3394 grams of sodium phosphate monobasic solution were combined to create phosphate buffer. A 0.1 M hydrochloric acid solution was used to bring the pH level down to 8.5, and 100 mL of water was added to make up the volume. A new NBD-Cl solution containing 5 mg mL^{-1} of methanol was made. The other solutions remained stable for at more than two weeks while being kept at 4 ºC.

Instrumentation and Chromatographic Conditions

Using a Shimadzu spectrofluorimeter Model RF-1501 fitted with a xenon lamp and 1 cm quartz cells, fluorescence spectra and measurements were obtained. The wavelengths for excitation and emission were chosen at 310 and 365 nm, respectively. We used a WTW pH 526 digital pH meter to measure the pH. A Shimadzu (Japan) LC 20 liquid chromatograph, which included a CTO 10 AC column oven, a SIL AT-HT autosampler component, an SPD-20A HT, and an LC-20AT pump, was used to conduct the HPLC analyses.

To achieve the most effective chromatographic separation, several combinations of mobile phase, column types, and stationary phase size were tested with varying flow rates and column temperatures. With a C18 Inertsil® (150 × 4.6 mm × 2.6 µm) analytical column at 30 ºC, chromatographic separation was accomplished isocratically using a mobile phase consisting of acetonitrile-0.1% o-phosphoric acid in water (80:20, v/v) at a flow rate of 1.0 mL min^{-1}.

Sample Preparation and General Procedure

Five milliliters of venous blood were drawn from a volunteer's peripheral veins (an informed permission form was obtained with ethical committee approval), placed in tubes with disodium EDTA, and centrifuged for ten minutes at 4500 × g. The resulting plasma samples were kept in storage at -20 degrees Celsius. The drug was extracted from the plasma samples by alkalinizing 1.0 mL of plasma with 500 µL 0.1 M NaOH, adding fampridine working solutions, and then extracting the solution into 5 mL of chloroform. After five minutes of moderate-speed vortex mixing, the contents were centrifuged for five minutes at 4500 × g.

The watery layer was thrown away. At 40 ºC, a spray of nitrogen was used to evaporate the organic layer until it was completely dry. After adding 500 µL of pH 8.5 phospate buffer, 500 µL of 5 mg mL^{-1} NBD-Cl solution, and 1 mL of water to the residue, the system was heated for seven minutes at 80 °C. After cooling in an ice batch, 0.2 mL of 1 N HCl solutions were used to acidify the mixture. After 30 seconds of vigorous mixing the solution with a vortex mixer, 20 µL of the derivatized sample was introduced into the HPLC apparatus.

RESULTS AND DISCUSSION

Derivatization Process

The conditions under which fampridine and NBD-Cl reacted were examined and improved for the reaction product's efficiency. Every parameter has been altered independently, while the others remained unchanged. The ideal reaction time, temperature, pH, kind of buffer, acetonitrile-water proportions, mole ratio of NBD-Cl/fampridine, concentration of HCl, and amount of acidification needed to halt the derivatization reaction were all established. Figure 2 illustrates the response of derivatization.

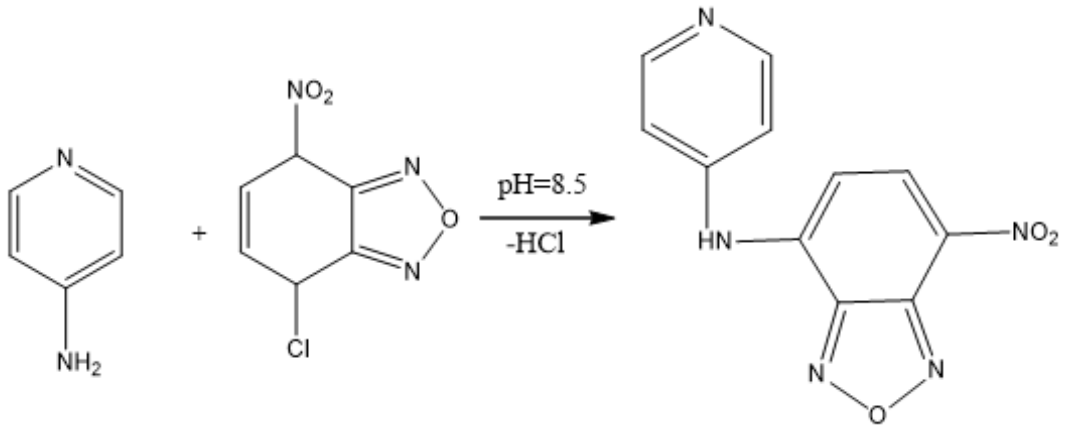


Fig. 2 – Derivatization reaction between fampridine and NBD-Cl.

Effect of pH

NBD-Cl is a nonfluorescent reagent that reacts with amino or thiol groups to become highly fluorescent. Furthermore, NBD-Cl offers a straightforward and accurate technique for identifying N-terminal amino acids. Prolyl peptides can be identified by taking advantage of the variations in fluorescence color and intensity.[23] The research of pH was limited to the range of 7–11 using phosphate buffer because it was observed that the fluorescence emission was only created in alkaline medium.[24] At pH 8.5, the suggested method's maximum absorbance was achieved.

Effect of time and temperature

The derivatization reaction was conducted at various temperatures and times to ascertain the ideal

temperature and reaction duration. The mixture was heated in a thermostatically controlled water bath at 80 ºC for seven minutes in order to create the fluorophore.

Effect of NBD-Cl concentration

The study examined the impact of the concentration of NBD-Cl on the derivatization reaction. 0.025 mmol (500 µL of 0.5% (w/v)) NBD-Cl solution was determined to be adequate to achieve the highest intensity.

Effect of acetonitrile to water ratio in derivatization medium

Various acetonitrile and water volumes were tested while maintaining constant medication, buffer, and NBD-Cl solution concentrations. Using a 1:3 acetonitrile to water ratio produced maximum peak area.

Stoichiometry of the reaction

Using Job's method of continuous variation, the molar ratio of NBD-Cl to fampridine in the reaction mixture was examined.[25] The reaction stoichiometry was determined to be close to a 1:1 ratio using equimolar solutions of fampridine and NBD-Cl. Peak regions indicate that there is neither a shortage nor an excess of the reagent in this stoichiometric ratio, indicating that all of the reagent has been utilized. To determine the ideal conditions for investigating derivatization reactions, all solutions were injected into an HPLC machine, and peak areas were measured. For at least 24 hours, derivatives prepared under the aforementioned circumstances stayed stable.

Effect of HCl concentration for acidification

The most effective results were obtained with 0.2 mL of 1.0 N HCl to generate NBD-OH to eliminate the surplus of NBD-Cl.

Chromatographic Process

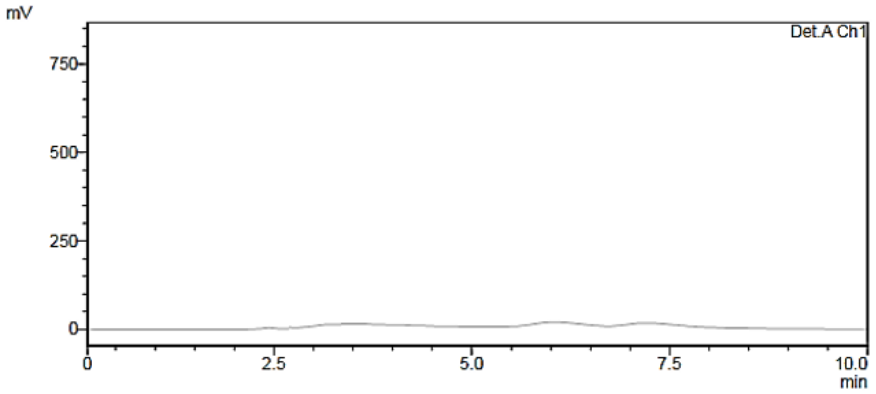
Using an isocratic elution technique and HPLC-FL as previously mentioned, a satisfactory separation of the derivatives and endogenous chemicals of plasma was achieved. Figure 3a-e shows representative chromatograms of the blank plasma, plasma samples spiked with 10 ng mL^{-1} fampridine, and plasma samples of the volunteer who took Ampyra® extended release tablets containing 10 mg fampridine at t_{max}. There was no evidence of interference with the components of the plasma. Fampridine has a retention time of 4.10 ± 0.02 minutes. The chromatographic system suitability parameters are shown in Table 1.

Table 1

Chromatographic system suitability parameters

Retention factor[a,*] (1–10)[b]	Resolution[a,*] (>2)[b]	HETP[a,*] (<1 mm)[b]	Tailing factor[a,*] (<2 mm)[b]	Asymmetry factor[a,*] (<2)[b]	Retention time (min)
7.23	3.64	0.08	1.2	1.1	4.10

*mean values of the parameters of all the points in calibration study are mentioned
[a]Average values
[b]Recommended value



(a)

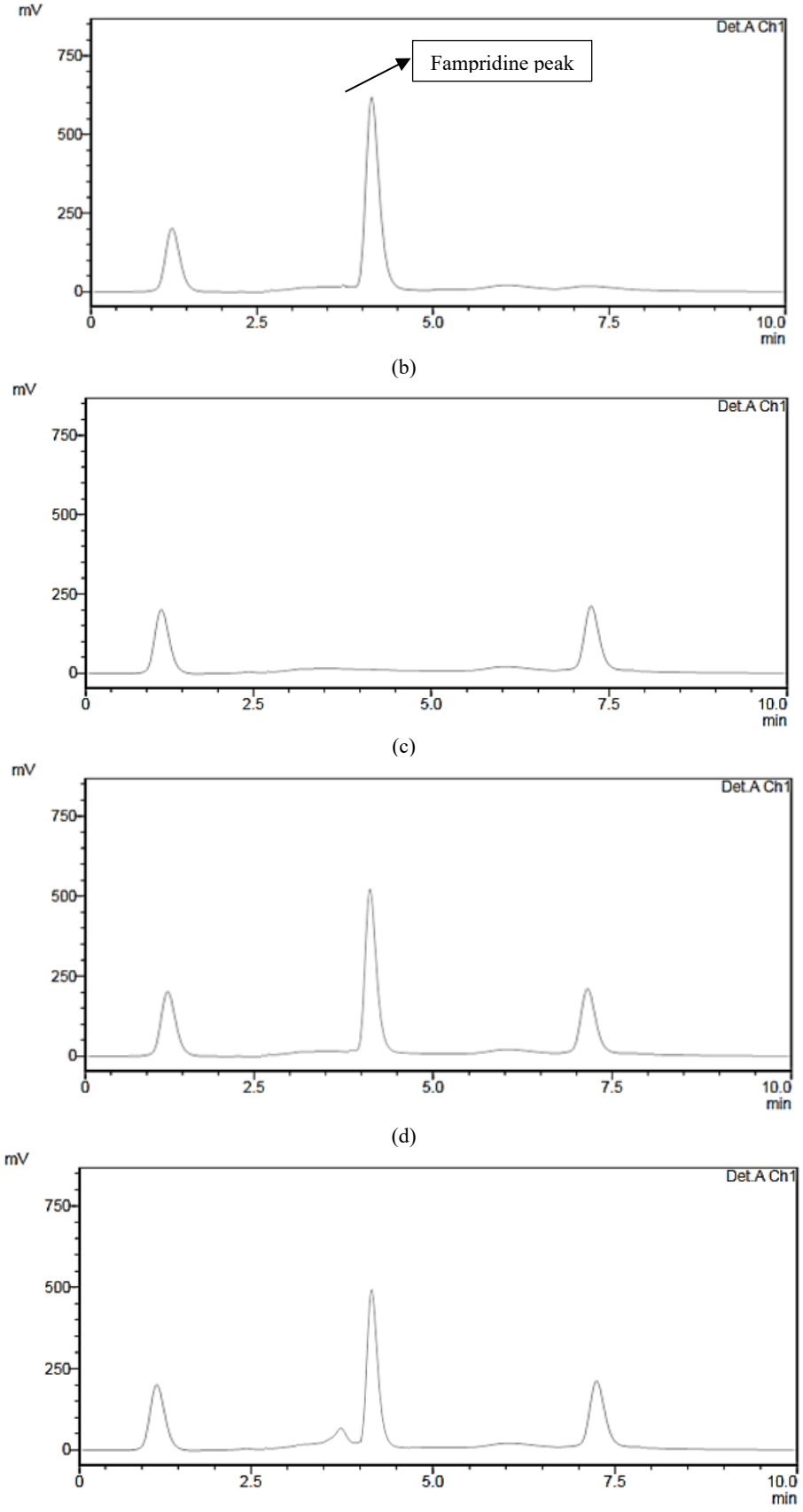


Fig. 3 – a) blank (aqueous medium); b) standard solution (10 ng/mL standard fampridine solution); c) blank plasma sample; d) 10 ng/mLfampridine spiked to plasma; e) plasma sample of volunteer after t_{mac}

Validation of the Method

Following the guidelines provided by the European Medical Agency (EMA), the procedure was validated.[26]

Linearity and sensitivity

Using a calibration curve in the range of 0.01–20 ng mL^{-1} of the drug (n = 5), the method's linearity was assessed. Five milliliters of fampridine plasma samples that had been spiked with different quantities of each working standard fampridine solution were analyzed to create calibration curves. Following that, the samples were put through the previously mentioned extraction, derivatization, chromatographic separation, and fluorometric detection procedures. Plotting the peak regions of the derivative against the appropriate fampridine concentrations allowed for the creation of calibration curves using linear least-squares regression analysis.

Table 2

Analytical parameters of the method

Parameters	Method
Concentration range [a] (ng mL^{-1})	0.01-20
Regression equation[b]	y=230170×+16667
Intercept± SD	230170± 280
Slope± SD	16667± 12
Correlation coefficient (r)	0.9996
LOD (ng mL^{-1})	0.003
LOQ (ng mL^{-1})	0.01

[a] Average of six determinatons

[b] $y=xC$ +b where C is the concentration in ng mL^{-1} and y is the peak area

The calibration curve (n = 5) derived from five points (0.01, 0.1, 1.0, 10.0, 20.0 ng mL^{-1}) has the following equation: y = 230170 x + 16667 (correlation coefficient = 0.9996), where x is the fampridine concentration and y is the peak area of the fampridine-NBD-Cl derivative.

LOD or LOQ = kSDa/b, where k = 3 for LOD and 10 for LOQ, was used to calculate the limit of detection (LOD) and limit of quantitaion (LOQ). SDa is the standard deviation of the intercept, and b is the slope. Table 2 provides a summary of the parameters for the analytical performance of the suggested approach.

Accuracy, precision and recovery

QC samples were determined at three concentration levels in order to evaluate accuracy and precision. QC samples were created in plasma and aqueous samples at three distinct concentrations (0.01, 1.0, and 20 ng mL^{-1}) that fall into the low, medium, and high concentration (n=3) categories. Recovery values and RME were used to express accuracy, whereas RSD was used to express precision. By extracting and derivatizing fampridine-spike plasma samples and comparing the peak regions obtained after derivatizing the same amounts of aqueous unextracted fampridine solutions, the 100% recovery of fampridine from plasma samples was investigated. Fampridine's mean absolute recovery was 99.52%. By comparing the amounts that are added to spiked and measured by the calibration curve, the mean relative recovery was determined to be 98.34%. For intraday precision and accuracy, three replicates of each concentration were assayed on the same day, and for interday precision and accuracy, three separate days were used. All of the intraday and interday assays had RDS values below 2.45%. Based on all of these findings, which are compiled in Table 3, good accuracy and precision were noted.

Table 3

Accuracy and precision of the method

Existant concentration (ng mL^{-1})	Added concentration (ng mL^{-1})	Found concentration (ng mL^{-1}) (Mean±SD[1])	Recovery (%)	RSD of recovery	RSD of intraday variation	RSD of interday variation
1	0.01	0.99 ± 0.04	98.01	1.43	1.53	2.13
	1	1.95 ± 0.06	97.50	1.57	1.84	2.45
	20	20.90 ± 0.07	99.52	1.07	1.26	2.02
Mean relative recovery = 98.34						

For each concentration n = 3

Robustness

As mentioned in the validation section above, robustness was evaluated by determining the QC samples at three concentration levels (n=3). The flow rate, column oven temperature, and the amount of acetonitrile and water phase in the mobile phase are the variables that are altered to gauge how robust the procedure is. Changes were made to the column

temperature from 30 ºC to 25 ºC and 35 ºC, the proportions of the mobile phase (acetonitrile-water solution) from 80:20 v/v to 85:15 and 75:25, and the flow rate from 1.0 mL min^{-1} to 0.8 and 1.2 mL min^{-1}. Peak area and resolution were not significantly impacted by these adjustments. Low RSD values demonstrate the method's resilience in Table 4.

Table 4

Robustness of the method

Condition	Value	Recovery %	RSD %
Flow rate mL min^{-1}	0.8	98.2	1.12
	1.2	98.4	1.04
Mobile phase composition (methanol:aqueous phase)	75:25	98.3	2.21
	85:15	99.6	2.06
Column temperature	25	99.5	1.21
	35	99.7	1.13

n = 3 for all QC sample levels

Stability

Three replicates and various storage settings were used to examine the stability of working standard fampridine solutions at QC levels. The experimental storage settings include 24 hours of dark, room temperature storage, 24 hours of autosampler conditions, and 1 month of refrigeration at 4 ºC. The recovery rates for the conditions under testing are 98.2%, 97.3%, and 98.6%, respectively. The recovery percentage numbers are higher than those from our earlier researchs.[27,28] Out of all these studies, the highest RSD percentage was 3.23%. It should be noted that fampridine was shown to be stable under all test setting.

Application of the Method to Pharmacokinetic Analysis

In order to determine the drug material in plasma for the pharmacokinetic research, the proposed approach was used. Fampridine (10 mg) was given orally as a single dosage to a male volunteer who was in good health and aged 30. On the first day, approximately 5 mL of venous blood samples were taken before the dosage and 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 7, 8, and 10 hours later. During the next five days, blood samples were taken once daily. As previously mentioned, the blood samples were converted to plasma. A chromatogram of the plasma sample taken 3.5 hours after the volunteer received a single oral dose of 10 mg fampridine is displayed in Fig. 2e. Until analysis, the samples were kept at –20 ºC.

Using the analysis performed by the suggested strategy, pharmacokinetic parameters were computed. The TOPFIT 2.0 pharmacokinetic and pharmacodynamic data analysis system was used to compute the area under the plasma concentration-time curves (AUC_{0-12}, $AUC_{0-\infty}$).[29] Figure 4 displays the fampridine plasma concentration-time curve following oral delivery of a single oral dose of 10 mg of medication. The pharmacokinetic parameters listed in Table 5 are the same as those that were discovered earlier.[30]

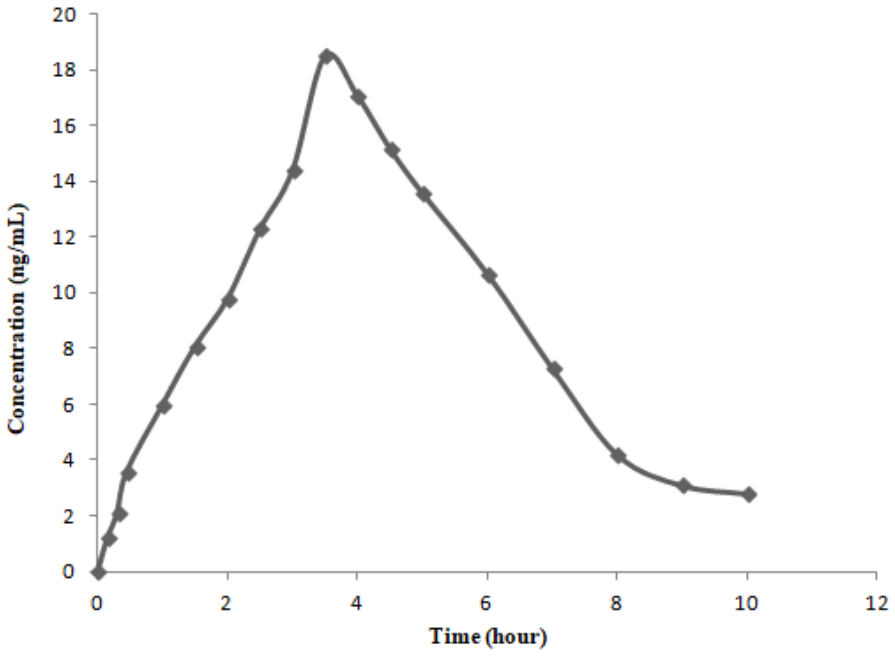


Fig. 4 – Pharmacokinetic curve of fampridine after administration of 10 mg dose orall.

Table 5

Pharmacokinetic parameters of fampridine after administration of single oral dose of 10 mg

Parameter	Found value
T_{max} [a](h)	3.5
C_{max} [b](ng mL^{-1})	18.56
$t_{1/2}$[c](h)	5.8
AUC^{d}_{0-10} (ng h mL^{-1})	41.6
$AUC^{d}_{0-\infty}$ (ng h mL^{-1})	48.8

[a]Time to maximum concentration
[b]Maximum concentration,
[c]Elimination half life,
[d]Area under the concentration-time curve

CONCLUSION

People with multiple sclerosis, a condition in which the nerves malfunction and can result in weakness, numbness, loss of muscular coordination, and issues with vision, speech, and bladder control, can walk more easily with the help of fampridine (dalfampridine). Since this medication is relatively new, research on drug-drug and drug-food interactions as well as side effects is necessary. The HPLC-FL approach that is being given is reproducible, sensitive, and economical. The drug substance's retention time is approximately 4.10 minutes, indicating that the analysis was completed in a very short amount of time. Nearly every drug laboratory can use highly fluorescent derivatives of fampridine with NBD-Cl to accurately identify the drug material fluorometrically using basic HPLC-FL. This is the first time fampridine has been detected fluorimetrically in the literature. The suggested approach is undoubtedly applicable to regular drug analysis in plasma as well as bioequivalence and bioavailability studies.

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