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The Creation and Validation of a New High-Performance Liquid Chromatography Method for Analyzing Pyridostigmine Bromide and its Degradation Products in a Pharmaceutical Formulation

*Sherif M. Fawzy[1, *], Mohamed A. Helal[2,3], Lobna M. Abdel-Aziz[4], Ismail Salama[3]*

[1]Pharmaceutical Chemistry Department, Faculty of Pharmacy, Sinai University, Kantara, Egypt
[2]Biomedical Sciences Program, Zewail City of Science and Technology, Giza, Egypt
[3]Medicinal Chemistry Department, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt
[4]Medicinal Chemistry Department, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt
**Corresponding author*

Correspondence:

Sherif M. Fawzy
sherif.fawzy@su.edu.eg

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ABSTRACT

A novel, rapid, sensitive, and validated high-performance liquid chromatographic (HPLC) technique, with high selectivity for stability indication, has been employed to determine pyridostigmine bromide and its degradation products (3-hydroxy-1-methylpyidin-1-ium bromide and 3,4-dioxo-3,4-dihydropyeidin-1-ium) in a pharmaceutical formulation. Pyridostigmine bromide and its degradation products were identified using combinations of acetonitrile and 0.01M acetate buffer (pH=6) in a ratio of 5:95 (v/v), through flow rates of about 1 ml/min at room temperature, employing a diode array detector. The linear range was determined to be 27-135 µg/ml. Our findings indicate that pyridostigmine bromide is unstable in alkaline conditions but more stable in acidic environments and susceptible to oxidation.

KEYWORDS: HPLC, DAD, UPLC, PYR.

1. INTRODUCTION

Pyridostigmine bromide (PYR) acts as a parasympathomimetic and works as a reversible inhibitor of cholinesterase [1-5]. Due to its nature as a quaternary amine, it exhibits low absorption in the gastrointestinal tract and cannot traverse the blood-brain barrier [6-7]. PYR can be utilized for muscle weakness treatment in individuals with myasthenia gravis and to counteract the adverse impacts of curariform drug toxicities. FDA has sanctioned the use of PYR for military purposes in combat scenarios, administering it as a pre-exposure prophylactic agent against nerve agent Soman to enhance survivability. Its utilization, notably during the initial Gulf War, has raised concerns about its potential contribution to Gulf War syndrome [8-12]. It is also used to treat orthostatic hypotension [13]. Also, Pyridostigmine plays a role in managing chronic axonal polyneuropathy [13].

As Pyridostigmine bromide is a quaternary ammonium compound with limited lipid solubility and a positive charge, it hinders its effective penetration of the blood-brain barriers, consequently limiting its influences on the central nervous system [14]. There is limited evidence suggesting any negative influences associated with the use of PYR in human treatment [15-17]. It is subjected to hydrolysis by cholinesterase and further metabolized by microsomal enzymes found in the liver. The main metabolite produced is 3-hydroxy-N-methyl

pyridine despite the idea that there is limited mention of any biological actions attributed to it [18, 24]. A few capillary electrophoresis approaches were reported for the assay of PYR [25] besides another method for assaying pyridostigmine with neostigmine in plasma using gas chromatography [26]. Many HPLC methods for the assay of PYR either alone or with other drugs in biological samples have been reported [27-31]. The pharmacokinetics and oral bioavailability of PYR in humans were investigated in the following study [32-34]. It's necessary to conduct stability studies by making forced degradation of PYR to ensure the method is stability-indicating [35-38].

Our study aims to renovate a new stability-indicating HPLC assay technique for accurately determining pyridostigmine bromide, intended for quality control and stability studies. The objective is to establish a precise and accurate method capable of effectively separating pyridostigmine bromide from its degradation products and impurities.

2. MATERIAL AND METHODS

2.1. Materials

Pystinon® was provided by Alexandria company for the pharmaceuticals and chemicals industry, Gamila Bohried Street, Al Awayed area, Alexandria, Egypt. Each tablet of it contains 60 mg pyridostigmine bromide, (Batch No. 4163004).

Acetonitrile used was HPLC grade (Fisher Scientific, NJ, USA). The water used was double distilled. Ammonium acetate, hydrochloric acid, hydrogen peroxide, sodium hydroxide, and acetic acid were analytically graded**.**

2.2. Instrumentation

The liquids chromatograph used in this study belong to a Hitachi La Chrom Elite L-2000 series, which included photodiode array detectors (model L-2455), an autosampler (model L-2200), a column oven (model L-2300), and a built-in degasser (model L-2130) pump. All equipment was manufactured by Hitachi La Chrom Elite in Tokyo, Japan. The column, measuring 150mm in length and 4.6mm in internal diameter, was constructed using stainless steel and filled with Inertsil ODS-3v (5 µm particle sizes, GL Sciences, Tokyo, Japan). The data capture was conducted using the EZChrom Elite software developed by Agilent Technologies.

2.3. Chromatographic Conditions

The HPLC-DAD mobile phase was produced by combining Acetonitrile and ammonium acetate buffer (0.01M) at a pH of 6, with a volumetric ratio of 5 parts Acetonitrile to 95 parts ammonium acetate buffer. The mobile phase was passed through a 0.45 µm disposable filter (Millipore Milford, MA). The rate of flow was 1 ml/min. All measurements were conducted at room temperature. The volume of the injection was 30 µl. Quantification was accomplished via a diode array detector. The DAD wavelength was configured to do a scan across the range of 200 to 300 nm. Pyridostigmine bromide was identified using positive electron spray spectrometry (UPLC-ESI (+)-Ms) detection. The samples were injected into the ESI source using a syringe pump at a flow rate of 0.7 ml/min, while the cone voltage was set to 700 V. The mass spectra were obtained within the 100-1000m/z range. The study was

conducted using a methanol solution consisting of 0.1 M ammonium acetate at a ratio of 65% methanol to 35% ammonium acetate, at room temperature.

2.4. Methods

2.4.1. Preparation and Separation of Degradation Products of Pyridostigmine Bromide

2.4.1.1. Preparation of the alkali-induced degradation products

500 mg of pyridostigmine bromide was mixed with 50 ml 1N NaOH. The mixture was refluxed for 2 hours and then neutralized with 1N HCL. The mixture was evaporated to dryness. Oily hygroscopic residue was obtained and evaluated as a degradation product of pyridostigmine bromide under alkaline media.

2.4.1.2. Preparation of the acid-induced degradation products

500 mg of pyridostigmine bromide was mixed with 50 ml 2N HCL. The mixture was refluxed for 10 hours and then neutralized with 2N NaOH. The mixture was evaporated to dryness. The oily hygroscopic residue was obtained and analyzed as a degradation product of pyridostigmine bromide under an acidic medium.

2.4.1.3. The stability of Pyridostigmine bromide on hydrogen peroxide.

A solution was prepared by dissolving 500 mg of pyridostigmine bromide in 50 ml of water (H2O). An aliquot of 0.4 mm of hydrogen peroxide with a concentration of 33% w/v was introduced into a solution of Pyridostigmine bromide. The resulting mixture was allowed to sit undisturbed for 6 hours, after which it was subjected to analysis to determine the presence of oxidative breakdown products of pyridostigmine bromide.

2.4.2. Standard Solutions and Calibrations

Stock standard solutions were made by dissolving 100 mg of pyridostigmine bromide in 100 ml of distilled water. The solutions were subsequently prepared by diluting the stock solution with water. The solutions were then kept at 4°C.

2.4.3. Calibration of HPLC Method

The standard solutions of pyridostigmine bromide were prepared by diluting the stock solution with distilled water to make solutions within a range of concentration 27-135 µg/ml. A Triplicate of 30 µl was delivered for each concentration and subjected to chromatography using the predefined chromatographic conditions. Afterward, the data representing the region of the peak were graphed about their corresponding concentrations.

2.4.4. Samples Preparation

Ten tablets of Pystinon® were accurately weighed and then ground. A properly measured amount of the powder, approximately corresponding to 60 mg of pyridostigmine bromide, was dissolved in 100 ml of pure water. The obtained sample solution was subsequently passed through Whatman filter paper No. 41. Additional dilutions were made using distilled water to achieve the appropriate calibration range for each chemical. The concentrations of pyridostigmine bromide were obtained by using the standard protocols indicated in the HPLC technique calibration. The produced sample solutions were kept at a temperature of 4°C.

2.4.5. Forced Degradation Studies of the Active Pharmaceutical Ingredient (API) and Tablets.

To determine if our new method is stable, forced degradation reports were conducted on Pystinon® tablets and (API) of pyridostigmine bromide by subjecting them to acid, base, and oxidative degradation conditions [38].

2.4.5.1. Acid degradation studies

An amount of 10 ml of the stock solution of pyridostigmine bromide and Pystinon® sample solutions were moved into 50 ml volumetric containers separately. Each solution was then combined with 10 ml of 2N hydrochloric acid and refluxed for 5 hours. After refluxing, the mixtures were cooled, diluted with distilled water up to 50 ml, neutralized with a base, and subsequently assayed.

2.4.5.2. Alkali degradation studies

An amount of 10 ml of the stock solution of pyridostigmine bromide and Pystinon® were each moved into separate 50 ml volumetric containers. To these solutions, 10 ml of 0.02N NaOH was added, then refluxed for 8 hours at 50°C. After refluxing, the mixtures were cooled, diluted with distilled water up to 50 ml, neutralized with acid, and then assayed.

2.4.5.3. Oxidative degradation with H2O2

An amount of 5 ml of a pyridostigmine bromide stock solution and Pystinon® sample solutions were each moved into separate 50 ml volumetric containers. Then, an amount of 0.4 ml of hydrogen peroxide (33% w/v) was added to each flask. The flasks were left at room temperature for 6 hours.

2.4.5.4. Solution stability

The pyridostigmine bromide and Pystinon® solutions were shielded from light and stored at room temperature for 48 hours to assess their stability within a brief timeframe. After undergoing different treatments, all solutions were filtered using a 0.45 µm syringe filtration disk before being introduced into the HPLC system.

3. RESULTS AND DISCUSSION

3.1. Identification of the degradant

When pyridostigmine bromide refluxed with (0.01N NaOH) at 50°C for 8 hours or (1N) hydrochloric acid refluxed at 100°C for 5 hours, [3-hydroxy n-methyl pyridine] (DG2) were the degradation products of pyridostigmine bromide in both acidic and alkaline media. While [3,4-dioxo-3,4-dihydropyridin-1-ium] (DG1) was observed as oxidative degradation of PYR when it reacted with 33% H2O2 for 6 hours at ambient temperature. The proposed way for the degradation of PYR in (0.01N) sodium hydroxides, in (1N) hydrochloric acids and in 33% H2O2 is shown in the following (Fig.1). The degradation products of pyridostigmine were recognized via the comparison between (LC-ESI (+)-MS) spectra of pyridostigmine bromide in authentic and tablet before and after exposure to acidic, alkaline media and H2O2. The structure of the degradation product can be identified according to its molecular weight and the degradation pathway is shown in the following (Fig.1).

3.2. Chromatographic method

3.2.1. HPLC method

The purpose of our work is to develop a novel HPLC method for the separation of pyridostigmine bromide and its degradation products, a thing that helps in the quantitation of pyridostigmine bromide in its dosage form and makes studying the stability of the drug more available. A good resolution chromatogram of pyridostigmine bromide, DG1, and DG2 is shown in (Fig 2). Also, chromatograms of oxidative and hydrolytic degradation products of pyridostigmine bromide have been shown in (Fig 3-5).

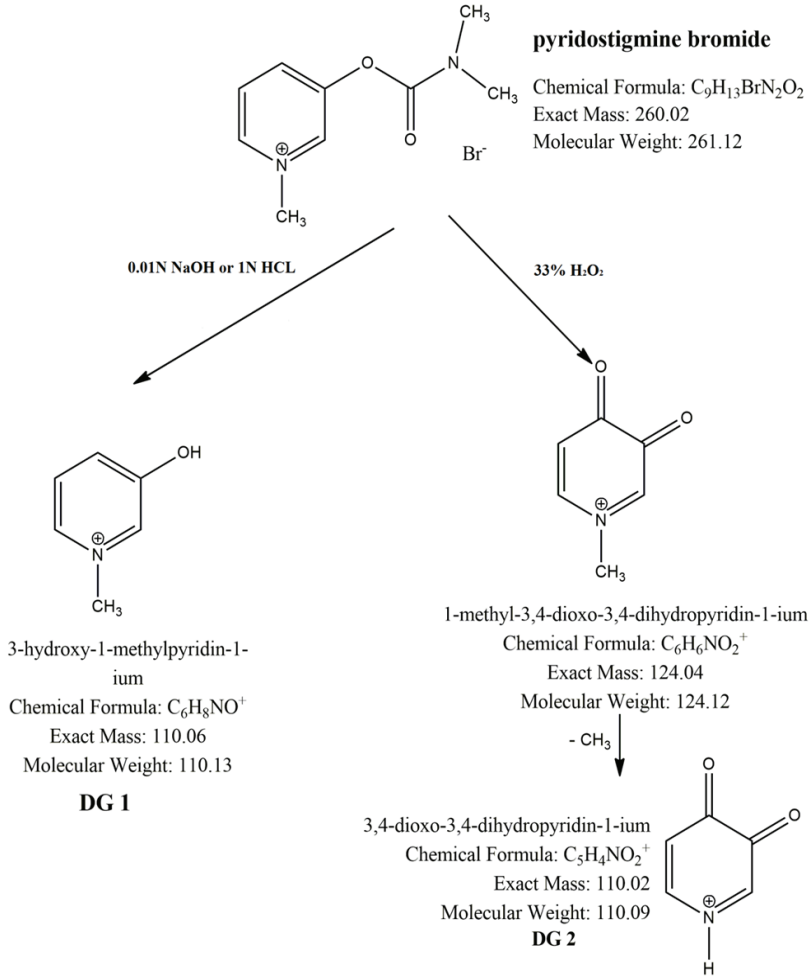

Fig. 15: Suggested pathway for the degradation of pyridostigmine bromide in 0.01N NaOH, 1N HCl and 33% H2O2.

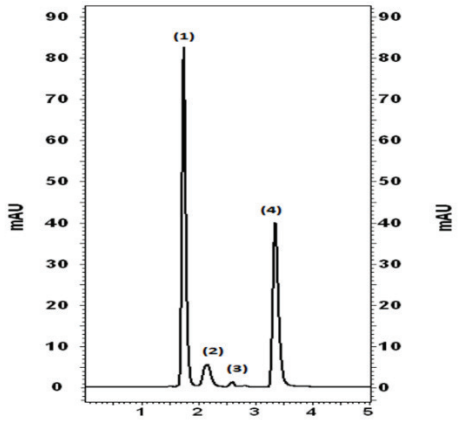

Fig. 16: HPLC chromatogram of pyridostigmine bromide and its degradation products (1) [3, 4 -dioxo-3, 4-dihydropyridin-1-ium] (2) [3hydroxy-1-methyl pyridine-1-ium], (3) [1-methyl-3, 4 - dioxo-3, 4-dihydropyridin-1-ium], (4) pyridostigmine bromide.

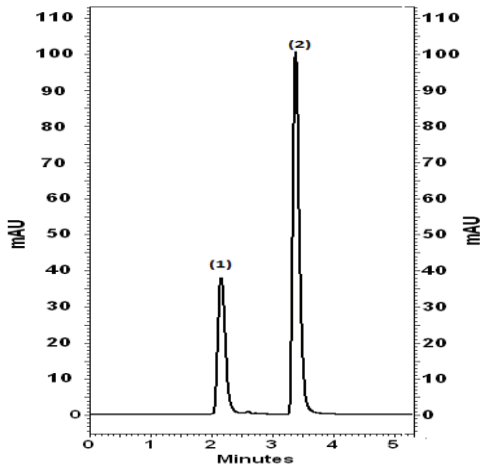

Fig. 17 HPLC chromatogram of pyridostigmine bromide in acidic media (1) [3hydroxy-1-methyl pyridine-1-ium] , (2) pyridostigmine bromide.

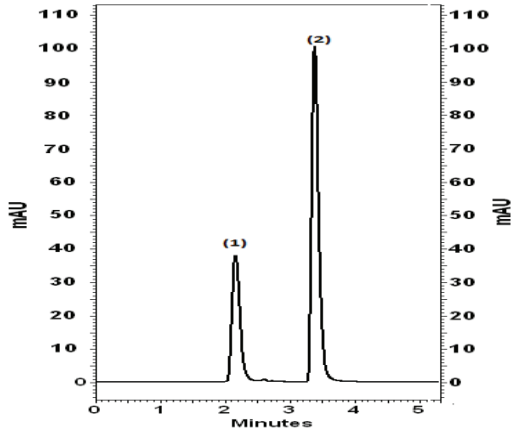

Fig. 18 HPLC chromatogram of pyridostigmine bromide in acidic media (1) [3hydroxy-1-methyl pyridine-1-ium] , (2) pyridostigmine bromide.

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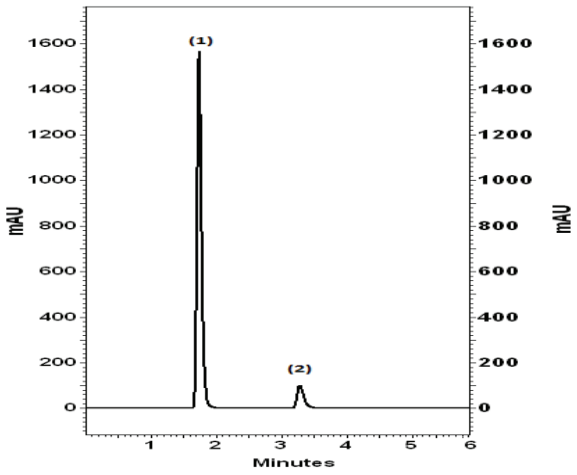

Fig. 19 HPLC chromatogram of pyridostigmine bromide in presence of 33% H2O2 for 6 hours (1) [3, 4 -dioxo-3, 4-dihydropyridin-1-ium], (2) pyridostigmine bromide.

The 3D of the peaks of pyridostigmine bromide and its degradation products was shown in (Fig.6), which checks the purity of the peak, by giving the full–scan absorption spectrum of the drug.

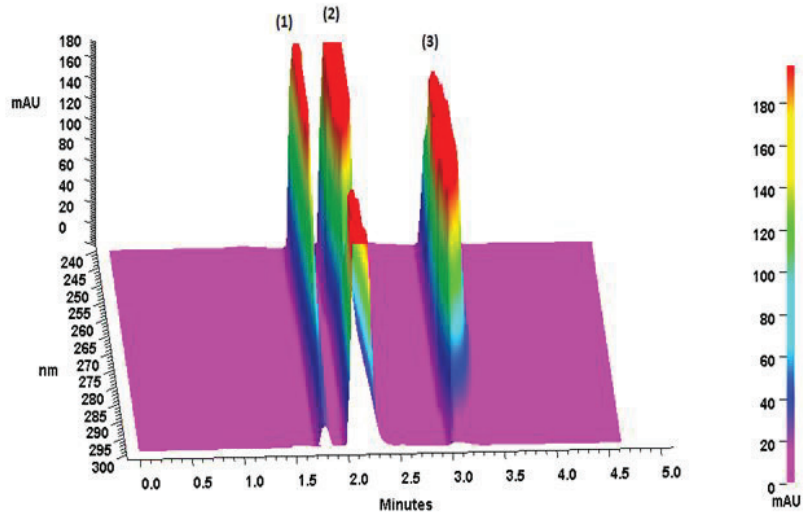

Fig. 20 DAD Spectrochromatogram of (1) DG2, (2) DG1 and (3) pyridostigmine bromide.

The developed HPLC methods of pyridostigmine bromide and its degradation product show good sensitivity and retention times. The development of the HPLC methods depends on testing variables at a time to give the best chromatographic conditions. The variables which tested were: flow rates, types and ratios of organic modifiers, temperature and wavelength. The method efficiency can be evaluated by main three responses: Tailing factor (T), Number of theoretical plates (N) and retention time (Rt). After trying several methods, the best results were by using acetonitrile and (0.01M) acetate buffer PH=6 in ratio (5:95 v/v) through flow rates of 1 ml/min at room temperature (Table 1).

Table 1: Effect of experimental parameters on the number of theoretical plate (N), Tailing factor (Tf) and retention time on pyridostigmine bromide, DG1 and DG2.

Parameter	No. of theoretical plates(N)			Tailing factor(T_f)			Retention time (R_t)		
	PYR	DG1	DG2	PYR	DG1	DG2	PYR	DG1	DG2
Ratio of organic modifier A/B									
85/15	880	608	1067	-	-	-	1.9	1.74	1.67
90/10	706	831	831	1.3	0.99	0.99	2.46	1.8	1.8
95/5	2685	1324	305	1.7	1.2	1.3	3.24	1.74	2.13
Flow rate (ml/min)									
1.5	2349	1237	2138	1.5	1.2	1.1	2.14	1.42	1.17
1.2	2576	1381	2641	1.7	1.3	1.3	1.45	1.7	1.76
1	2685	1324	3050	1.7	1.2	1.3	3.24	1.7	2.13
0.8	2865	1324	3551	1.8	1.3	1.4	2.65	1.4	1.8
Temperature (C°)									
25	2685	1324	3050	1.7	1.2	1.3	3.24	1.74	2.13
30	2812	1590	3195	1.7	1.3	1.2	3.19	1.74	2.1
35	2936	1702	3214	1.7	1.36	1.29	3.14	1.73	2.09
40	2917	1695	3202	1.8	1.3	1.3	3.09	1.72	2.0

3.2.1.1. Selection of the suitable wavelength:

The best wavelength obtained (after trying several wavelengths ranging from 200 nm to 300 nm using a diode array detector) was 270 nm. This wavelength gives the highest sensitivity and acceptable absorbance of pyridostigmine bromide and its degradation product.

3.2.1.2. Selection of the suitable temperature:

Many temperatures were tried to obtain the best temperature for the method. The obtained results are shown in (Table1).

3.2.1.3. Mobile phase composition:

Many trials have been applied by changing the mobile phase by trying different types and ratios of organic modifiers, the flow rate and the pH to obtain the best separation condition. The obtained results are presented in (Table 1).

a) Type of Organic Modifier

Several trials were applied using methanol and acetonitrile. Acetonitrile was the organic modifier of choice which gives good separation with high resolution.

b) Ratio of Organic Modifiers

Different ratios of organic modifier and acetate buffer have been applied, using different pH values. After several trials pH = 6 was the best value of the acetate buffer with 0.01 M concentration. Ranges of the ratio of organic modifiers from 80% acetonitrile to 95% have been tried. The ratio below 90% shows no separation, while the 95% ratio shows the best separation since it shows acceptable T, N and good resolution, as shown in Table 1.

c) Effects of Flow Rate

Several flow rates have been tried to reach to the fact that N, Rt, T and flow rate 1ml/min was optimal for separation.

3.3. Validation of the Methods

3.3.1. Linearity

The linearity has been evaluated using a series of different concentrations of pyridostigmine bromide. Then, the curve has been plotted between the area under the curve against concentration (µg/ml) to calculate the regression coefficient and slope of the curve. Five concentrations were chosen, showing that the best regression coefficient is ranging between 27-135 µg/ml (Fig.7). We repeated each concentration three times and the results area under the curve plotted against concentration to get the linearity equation and other linearity parameters shown in Table 2.

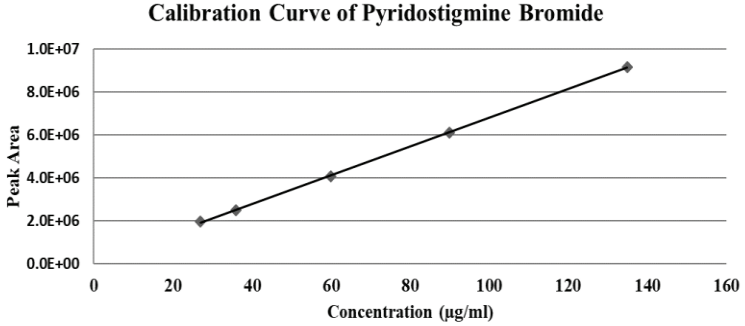

Fig. 21 Calibration curve for determination of pyridostigmine bromide using HPLC.

Table 2: Calibration curve data for pyridostigmine bromide.

Regression Parameters	Pyridostigmine
Regression coefficient (r)	0.9998
calibration range (µg/ml)	27-135
Detection limit (LOD) (µg/ml)	2.19
Quantification limit (LOQ)(µg/ml)	6.64
Slope ± SD	$6.6 \times 10^4 \pm 5.5 \times 10^2$
Confidence limit of the slope [a]	$68 \times 10^4 - 6.5 \times 10^4$
Intercept ± SD	$1.19\times 10^5 - 4.4\times 10^4$
Confidence limit of the intercept [a]	$2.19\times10^5 - 1.9\times 10^4$
Number of points	5

3.3.2. Range

To make sure that the results are accurate, precise and linear, we estimate the calibration range depending on the concentration of the compound present in the pharmaceutical product. The data of the calibration curve has been provided in Table 2.

3.3.3. Limit of detection (LOD) and limit of quantitation (LOQ)

Following the findings of the International Conference on Harmonies (ICH) [39], the method used for defining the detections and quantitation limits relied on the standard deviation (S.D.) of the responses and the slopes, as suggested by (ICH). The theoretical values were validated empirically and are presented in Table 2.

3.3.4. Precision

Repeatability of the method was achieved by using three different concentrations at three different levels for pyridostigmine bromide. The samples were then analyzed three times within the same day using our purpose HPLC method (intra-day) and on three different days (inter-day). The obtained results are presented in Table 3. The improved approach was determined to be accurate, as the coefficient of variation (C.V.) values were less than 2%.

Table 3: Intra-day and inter-day precision of pyridostigmine bromide.

Drug	Theoretical concentrations (µg/ml)	Intra-day precision Recovery%	(C.V.%)	Inter-day precision Recovery%	(C.V.%)
Pyridostigmine bromide	27	100.3	0.37	100.35	0.82
	60	98.6	0.93	100.7	0.52
	135	101.08	0.38	99.64	0.29

3.3.5. System Suitability Tests

To make sure that the method of separation is good and gives acceptable results, system suitability test parameter must be applied. The system suitability parameters were examined [40] (namely resolution, selectivity factor (α), capacity factor (K`), number of theoretical plates (N) and tailing factor (T) as shown in Table 4).

3.3.6. Accuracy

The accuracy of the method was performed by the standard addition techniques; this is applied by the addition of different known quantities of pyridostigmine bromide authentic to known concentrations of Pystinon® tablets. The obtained results were compared to the expected results and presented in Table 5. The good recoveries of the standard addition method suggest good accuracy of the proposed method.

Table 4: System suitability results of the HPLC method developed for analysis of pyridostigmine bromide, DG1 and DG2.

Parameters	Pyridostigmine bromide	DG1	DG2
Resolution (Rs)	3.82		2.05
Selectivity (α)	2.15		1.55
Symmetry factor (T)	1.7	1.2	1.3
Capacity factor (K`)	1.94	0.9	0.58
Number of theoretical plates (N)	2685	1324	3050
HETP (cm/plate)	0.003	0.011	0.004
Peak purity	1	0.97	0.74
3-point purity	0.97	0.99	0.987
Retention time	3.24	2.13	1.74

Table 5: Application of the standard addition technique for the pharmaceutical preparation (Pystinon® tablets).

No.	Claimed taken (µg/ml)	Authentic added (µg/ml)	Claimed area	Found area	Recovery%
1	30	18	3327697	3347065	100.58
2	30	42	4931972	4917930	99.71
3	30	60	6135107	6125106	99.83
Mean%					100.04
S.D					0.0046
RSD%					0.46

3.3.7. Robustness

The robustness of the method was determined by measuring different parameters (flow rate, temperature and organic modifier) and the results of pyridostigmine bromide, DG1 and DG2 were shown in Table 1.

3.4. Analysis of Tablets

The developed HPLC method was applied for the determination of pyridostigmine bromide in its pharmaceutical dosage form. Recoveries were calculated using external regression equations. Satisfactory results of accuracy and precision were obtained and indicated by the recovery, SD and RSD values (Table 5).

4. CONCLUSION

It is very important to study the pharmaceutical product quality to ensure patient safety. The safety and efficacy of the drug may be affected by the presence of impurities or degradation products. The study of drug stability is vital to ensure the delivery of safe and effective doses to patients.

The proposed HPLC method provides a sensitive, simple, accurate, precise, linear, repeatable, and robust analysis for the determination of pyridostigmine bromide in the presence of it is impurities and its degradation product in the pharmaceutical dosage form. Pyridostigmine bromide was found to be unstable in alkaline media while it is more stable in

acidic media. Pyridostigmine bromide should be kept in tightly closed bottle as it is easily oxidized.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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