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Simultaneous estimation of Pyridoxine HCl and FMOC-Leucine using derivative and chromatographic approach in pharmaceutical dosage form

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Abstract: This work developed a fast and simple method for quantifying two important amino acids, Pyridoxine Hydrochloride and Leucine, to treat obesity. In the current study, the active hydrogen of a free amino group at Leucine was only replaced by non-polar group using N-(9-Fluorenylmethoxycarbonyloxy) succinimide (FMOC-OSU) as a derivatizing agent, resulting in conversion of Leucine to FMOC-Leucine derivative to make an analyte less reactive, volatile, and thus improve its chromatographic behavior. The aim of the present study is to develop accurate, precise, and selective first order UV-Visible spectroscopic and HPLC methods for the simultaneous estimation of Leucine and pyridoxine HCl and to validate these methods as per International Conference on Harmonization (ICH) Q2 (R1) guidelines. In the developed method the correlation co-efficient was found to be 0.999; 0.999 and 0.995; 0.997 for FMOC-leucine and pyridoxine HCl respectively. The %RSD for accuracy was found to be less than 2. The detection limit and quantification limit for validation in UV-Visible spectroscopy and HPLC for Pyridoxine HCl and FMOC-Leucine were found under limit. The methods were also found to be precise and robust (% RSD= < 2%) and % assay of the marketed formulation containing both the drugs was found to be 98.6%; 98.7%; 99.2%; 99.3% for Pyridoxine HCl and FMOC-Leucine respectively. Comparison between both the methods was done by one-way ANOVA using % assay values. There was not any significant difference found. Both the methods can use for simultaneous estimation of Leucine and Pyridoxine HCl.

Keywords: Pyridoxine HCl; FMOC-Leucine; derivatization; UV-visible spectroscopy; HPLC; validation.

1.Introduction

Amino acids, a class of biologically active compounds, play a crucial role in human nutrition. Leucine (see supporting information Figure S1), 2-Amino-4-methylpentanoic acid, and/or its metabolites succour muscle during contraction by increasing intracellular calcium, as results activate proteins such as mTOR (mammalian target of Rapamycin) which induce the muscle protein synthesis. In contrast to

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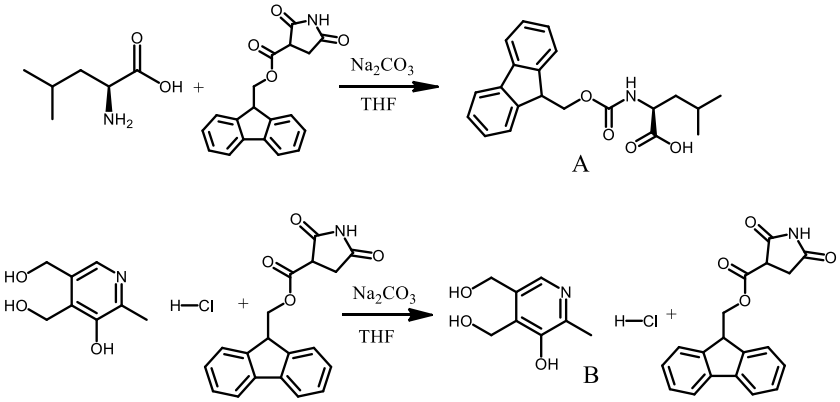
muscle contraction, however, Leucine probably does so in all cells rather than localized to skeletal muscle. It's indicates that it is able to averts the breakdown of muscle proteins that occur after trauma [1-2]. Pyridoxine (see supporting information Figure S1), 4,5-Bis(hydroxymethyl)-2-methyl pyridine-3-ol or Vitamin B_6, principally in its biologically active coenzyme form, pyridoxal 5'-phosphate, is involved extensively in biochemical reactions, like the amino acids and glycogen metabolism, the synthesis of several neurotransmitters, and the synthesis of hemoglobin, sphingomyelin, and other sphingolipids [3-4]. Interaction of these amino acids (Leucine and Vitamin B_6) also attenuates adiposity. Pyridoxal phosphate inhibits the inflow of Ca^{2+} adipocytes *in vitro*, resulting in significant decreases in the expression and activity of adipocyte fatty acid synthesis. Moreover, synergistically Leucine and Pyridoxal phosphate inhibit adipocyte triglyceride accumulation [5-6].

The combined form of Leucine and Pyridoxine Hydrochloride (HCl) in the tablet dosage form is approved for the treatment of obesity [7]. The expansion of a selective, rapid, reliable and accurate method of evaluation to assess the quality of the dosage form for regulatory purposes is of ongoing interest. Amino acids are generally analyzed after their derivatizationto make an analyte less reactive, volatile, and thus improve its chromatographic behavior [8].

The reverse phase high-performance liquid chromatography (RP-HPLC) with derivatization is preferred to get a simple, short, and low-cost method. Typical reagents for derivatization are N-(9-Fluorenylmethoxycarbonyloxy) succinimide (FMOC-OSU), Phenylisothiocyanate (PITC), 9-fluorenylmethyl-chloroformate (FMOC-Cl). Each of these reagents has particular advantages and limitations. Consequently, derivative methods have a long history in the detection of UV amino acids. Since many amino acids contain primary amino groups or (NHR) in their structures, a selective derivatizing reagent is used for the reaction [9-10]. In the current study, during derivatization it was observed that only active hydrogen of polar functional groups NH_2 in Leucine gets replaced by non-polar groups (FMOC-OSU) Figure 1, while the presence of tertiary nitrogen in Pyridoxine HCl, in its active form, does not allow it to form the derivatized product. Finally, UV spectrometry and the RP-HPLC method were developed for the analysis of FMOC-Leu and Pyridoxine HCl and were validated.



(A) – Derivatized FMOC-Leucine
(B) - No formation of derivatized product in Pyridoxine HCl

Figure 1. Derivatization reaction of Leucine and Pyridoxine HCl

2. Experimental

2.1. Materials and Methods

A UV–Visible spectrophotometer (2450 Shimadzu, software UV Probe 2.21) with a spectral bandwidth of 1 nm was employed for all spectroscopic measurements, using a pair of 10 mm matched quartz cells. Methanol (MeOH) was used as a solvent for UV-visible spectrophotometric determination of FMOC-Leucine (FMOC-Leu) and Pyridoxine HCl.Waters HPLC (Separation module 2695) chromatographic system equipped with UV detector Zorbex C18 (250X 4.6 mm, 5 µL) thermostatic column compartment connected with LC program software, consisting of pump, auto-sampler, and auto-injector. A nylon filter of 0.22 µm from Merck Millipore was used for filtration. BT ultra sonicator 48,

Millipore vacuum filter pump (XI 5522050), Shimadzu digital weighing balance (ATX 224), and digital systronic pH meter (802) were used for the development of the method.

The Pharmaceutical grade working standards of Pyridoxine HCl and Leucine was procured as a gift sample from Omgene life science Pvt. Ltd (Vadodara, GIDC, Gujarat, India). Pyridoxine HCl and Leucine tablet formulation (7.5 mg and 550 mg respectively) were used of Kg low, marketed by Indico Remedies LTD, Santacruz (E), Mumbai 400 098, India.The HPLC grade acetonitrile (ACN), MeOH, and orthophosphoric acid (OPA) were procured from Fisher Scientific, Mumbai, India while analytical grade HCl, sodium hydroxide, and hydrogen peroxide from Merck (Merck Euro-lab, Fontenay-sous-Bois, France). FMOC-OSU was obtained by C.S. BIO Ltd (Shanghai, China) and double distilled water was used throughout the analysis.

2.2. Preparation of Derivatized Products (FMOC-Leu)

For preparation of derivatized product, 2.6 gm of sodium carbonate (3 eq.) was dissolved in 10 mL of water with continuous stirring and then solution was cooled at 0-10 ^{0}C. Upon cooling 1 gm of the Leucine (1eq.) was added with continuous stirring. In another solution, 2.57 gm of FMOC-OSU was dissolved in 10 mL of tetra hydro furan (THF) and was mixed in a cooled solution of sodium carbonate with stirring. The excess of THF was needed to be distilled off and then the solution was diluted with 10 mL of ethyl acetate to form sodium salt. The pH of the resulting solution was adjusted between 3-4 by adding a solution of water and acid in ratio 1:1 v/v in a separating funnel. In the upper (acetate) layer product was appeared while the lower (water) layer was separated and was washed several times with ethyl acetate and then with brime and sodium sulfate solution. Finally, the solid product was treated with 5 mL of diethyl ether to remove non-polar impurity and un-reacted FMOC-OSU. The pure solid at the end was confirmed by thin-layer chromatography.

2.3. Preparation of Solutions

2.3.1. Preparation of Stock solution of FMOC-Leu and Pyridoxine HCl (100 ppm)

Initially, the solution of 1000 ppm (1000 µg/mL) was prepared by taking 100 mg of FMOC-Leu and Pyridoxine HCl as working standards into 100 mL volumetric flask, diluted with 40 mL of MeOH, and was sonicated. The solution was then cooled at room temperature and volume was adjusted with diluent up to the mark. Further, from the resulting solution, 2.5 mL solution was diluted up to 25 mL with the diluents, for a solution of the strength of 100 ppm (100 µg/mL) as a working stock solution.

2.3.2. Selection of Wavelength

For UV-visible spectroscopy, the **s**tandard solution of FMOC-Leu and Pyridoxine HCl were scanned in the range of 200-400 nm separately. Data were obtained by overlay spectra of both drugs where FMOC-Leu and Pyridoxine HCl show absorption maxima at 251 nm and 299 nm respectively for first-order derivative spectroscopy. The overlain spectra of the first-order derivative confirm that Pyridoxine HCl show zero-crossing at 251 nm, while FMOC-Leu showed zero-crossing at 299 nm at the ZCP of Pyridoxine HCl, FMOC-Leu showed a first derivative absorbance Figure 2, whereas, at the ZCP of FMOC-Leu, Pyridoxine HCl showed a first derivative absorbance. The detection of RP-HPLC was carried out at different wavelengths best response was achieved at 220 nm and 285 nm in Water: ACN (see supporting information Figure S2). Finally, for the detection, the wavelength selected was 285 nm.

Simultaneous estimation of Pyridoxine HCl and FMOC-Leucine

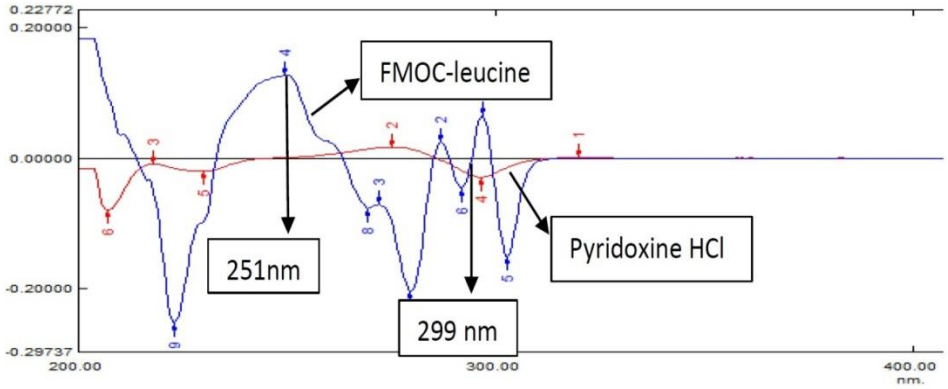


Figure 2. Overlay spectra of FMOC-LEU and Pyridoxine HCl showing ZCP

The two series of calibration curves at varying concentrations were prepared for both drugs. For preparation of sample solution, 100 µg/mL stock solution of Pyridoxine HCl and FMOC-Leu in range of (0.2, 0.4, 0.6, 0.8, 1.0 mL) and (1.4, 2.9, 4.3, 5.8, 7.3 mL) was pipette out and individually was diluted to 10 mL using MeOH as diluent, providing concentrations range of (2, 4, 6, 8, 10 µg/mL) and (14.6, 29.3, 43.98, 58.6, 73.3 µg/mL). Thus further absorbance of the above-prepared solution was noted in Figure S3 (see supporting information) using a spectrophotometer.

2.3.3. Analysis of Marketed Formulation

The mixture was prepared by mixing drugs with placebo according to tablet composition of kg low. A portion of powder, equivalent to derivatized 73.3 mg of Leucine and 1 mg of Pyridoxine HCl was accurately weighed and transferred into a 100 mL volumetric flask diluted with MeOH and was sonicated for 5 min to get 10 µg/mL and 733 µg/mL of Pyridoxine HCl and FMOC-Leu. From the above stock solution, 4mL was diluted to 10 mL using a diluent to get a solution of 4µg/mL and 293.2µg/mL of Pyridoxine HCl and FMOC-Leu respectively. Finally, the solution was diluted by taking 1mL of solution into 10 mL of diluents to get 0.4 µg/mL and 29.3 µg/mL of Pyridoxine HCl and FMOC-Leu respectively. Finally, the solutions were scanned at 299 nm for UV and 285 nm for RP-HPLC. The applicability of the method was confirmed by determining the %assay of the marketed formulation of Pyridoxine HCl and FMOC-Leu by UV-visible spectrophotometry and RP-HPLC methods.

2.3.4. Preparation of Mobile Phase for RP-HPLC method

The mobile phase was composed of 0.1% OPA in water and ACN in the ratio 50:50 % v/v. Initially, the filtered solution, through a 0.22 µm membrane filter, was sonicated for 15 min to degas it. Both the drugs were separated and eluted in anisocraticprogram.

2.3.5. Chromatographic Conditions RP-HPLC

The mobile phase is composed of 0.1% OPA in water and ACN in the ratio of 50:50% v/v. The flow rate of sample elution was secured at 1.0 mL/min with an injection volume of 10 µL. The analysis was performed at ambient temperature, and the detection was carried out at 285 nm Figure 3.

Figure 3. Chromatogram of mixture in MPA: 0.1% OPA in Water MPB: ACN at 285nm in Zodiac;4.6 ID *250mm; C18; 5µm

2.3.6. System Suitability Test

The various system suitability parameters like retention time, tailing factor, and theoretical plates for optimized standard mixture chromatograms are tabulated in Table 1. For all system suitability injections, retention time was found to be 3.4 min and 14.3 min for, tailing factors were 1.47 and 1.49 and theoretical plates were greater than 2000 for Pyridoxine HCl and FMOC-Leu respectively, suggesting system suitability for Pyridoxine HCl analysis (see supporting information Figure S4).

Table 1. System suitability of Pyridoxine HCl and FMOC-Leu

Parameters	Pyridoxine HCl	FMOC-Leu
Theoretical plate	2800	62997
Tailing factor	1.47	1.49
Retention time	3.4	14.3
Area	264082	647667
Resolution	0.00	55.7

2.3.7. Limit of Detection (LOD) and Limit of Quantification (LOQ)

As per formula it can be calculated as
Formula - LOD = X × SD / slope
LOQ = X× SD / slope
Where
X= 3.3 (LOD)
X= 10 (LOQ)
SD = standard deviation

The result for the same is discussed in section 3.4.9.

3. Results and discussion

3.1. Characterization of derivatized FMOC-Leu

The derivatized FMOC-Leu obtained after work-up was white solid product was tenderly soluble in water and freely soluble in ACN, MeOH, and ethanol. The melting point of the derivatized product was observed 154-156 ^{0}C and R_f 0.7 in the ratio of (1:1) ethyl acetate and hexane. The IR spectra of FMOC-

Leu show peaks at 3333 (NH str), 3040 (Ar-CH str), 1677 (-COO). In the mass spectra the *m/z* value was observed at 354.2 and 355.4 (see supoorting information Figure S5).

3.2. Analysis of Marketed Formulation

The present method was applied for the detection of Pyridoxine HCl and Leucine in marketed tablet dosage forms of 7.5 mg and 550 mg respectively. The percentage assay results in UV-visible spectroscopy and RP-HPLC for pyridoxine HCl were found to be 98.6% and 98.7 % with the amount found were up to 7.39 mg and 7.40 mg while for Leucine results for percentage assay were found to be 99.2% and 99.3% with the amount found were up to 545.6 mg and 546.1 mg. The results are summarized in Table S1and Figure S6 (see supporting information).

3.3. Chromatographic Conditions RP-HPLC

The mobile phase is composed of 0.1% OPA in ACN and water in the equal ratio (50:50)% v/v. The flow rate of sample elution was secured at 1.0 mL/min with an injection volume of 10 µL. The analysis was performed at contexture temperature 25 °C, and the detection was carried out at 285 nm. The RT for the drugs Pyridoxine HCl and FMOC-Leu was observed at 3.4 and 14.3 min respectively. The data is tabulated in Table 2.

Table 2. Optimized chromatographic condition

Column	Zorbex; C18; 250 mm X 4.6 mm, 5 µm
Flow Rate	1.0 mL/min
Wavelength	285 nm
Column Temp.	40 °C
Auto sampler Temp.	25 °C
Rum Time	10 min
Retention Time (min)	Pyridoxine HCl - 3.4 FMOC-Leu - 14.3
Mobile Phase	0.1 % OPA + ACN: Water (50:50)
Tailing Factor (<2)	Pyridoxine HCl – 1.47 FMOC-Leu– 1.49
Resolution (>2)	Pyridoxine HCl – 0.00 FMOC-Leu– 55.86
Theoretical Plate (>2000)	Pyridoxine HCl – 2800 FMOC-Leu– 62997

3.4. Method Validation for UV-Visible Spectroscopy and RP-HPLC:

The developed UV-visible Spectroscopy and RP-HPLC method was validated as per ICH Q2 (R1) guidelines. [11].

3.4.1. Linearity

Linearity response was determined by 5 independent levels of the calibration curve in the range of 2-10µg/mL and 14.6-73.3 µg/mL for Pyridoxine HCl and FMOC-Leu respectively in UV-visible spectroscopy Table 3A and RP-HPLC Table 3B.

Table 3. Linearity Concentration in UV spectroscopy **(A)** and HPLC **(B)** of Pyridoxine and FMOC-Leu

A

Concentration (ppm)	Response at 229 nm	Concentration (ppm)	Response at 251 nm
2	0.0101	14.6	0.0335
4	0.0139	29.3	0.0588
6	0.0178	43.9	0.0806
8	0.219	58.6	0.1016
10	0.0258	73.3	0.1244

B

Pyridoxine HCl			FMOC-LEU		
Conc. (µg/mL)	Peak Area	Mean ± SD	Conc. (µg/mL)	Peak Area	Mean ± SD
2	163513	163210.2±1655.456	14.6	383319	387209±6654.628
4	210442	210168.6±1018.066	29.3	516574	512912.8±587.408
6	263418	263872.6±1040.896	43.9	645621	641912±5748.62
8	312776	311317.4±1659.795	58.6	763263	766654±6370.166
10	352564	343477.8±21520.48	73.3	857150	856399±2370.448

The overlaid spectra and a calibration curve of Pyridoxine HCl and FMOC-Leu were constructed by plotting absorbance v/s concentration and area v/s concentration for UV and RP-HPLC respectively**.** The retention time in RP-HPLC was observed 3.4 min and 14.3 min for Pyridoxine HCl and FMOC-Leu was respectively. The R^2 value in UV was found to be 0.999 and 0.999 Figure S7 while for RP-HPLC 0**.**997 and 0.995 Figure S8 for Pyridoxine HCl and FMOC-Leu respectively (see supporting information).

3.4.2. Precision

There exist three different levels for precision like system, method, and intermediate precision for evaluation. Each level has been explored by replicate injections of 100 ppm (100%) concentration of Pyridoxine HCl and FMOC-Leu.

3.4.3. Repeatability

The data for repeatability of absorbance measurement (UV-visible spectroscopy) for Pyridoxine HCl (6 µg/mL) and FMOC-Leu (43.9 µg/mL), shows the % RSD 0.798 and 0.254 while in RP-HPLC it was observed 0.2068 and 0.2234. The parameters were within the acceptable criteria tabulated in Table 4. Hence it could be said that the methods provide satisfactory results on repeated sampling.

Table 4. Summary of validation parameters

Parameter		UV Spectroscopy		HPLC	
		FMOC-Leu	**Pyridoxine HCl**	**FMOC-Leu**	**Pyridoxine HCl**
Linearity (R^2)		0.999	0.999	0.995	0.997
Accuracy (%Recovery)		99.56	98.53	99.7	98.3
Precision (%RSD)	Repeatability	0.254	0.798	0.223	0.206
	Intraday precision	0.641	0.455	0.455	0.455
	Interday precision	0.431	0.828	0.828	1.177
LOD and LOQ		0.380 & 1.153	0.252 & 0.765	2.334 & 7.073	0.0486 & 0.147
Robustness (%RSD)		0.0866	0.0178	Wavelength	
				0.351	1.092
				Flow rate	
				0.351	0.782
% Assay (% Label claim)		99.2	98.6	99.36	98.7

3.4.4. Intraday and Interday Precision

For intraday precision in RP-HPLC, three replicates of three concentrations of a standard solution of both the drug. Total nine determinations were analyzed on the same day and Peak areas were measured and %RSD was calculated 0.27%-0.76% and 0.270%-0.761% for Pyridoxine HCl and FMOC-Leu respectively (see supporting information Table S2). Similarly, for inter-day precision (UV-visible spectroscopy), the % R.S.D was found to be 0.245-0.595% and 0.690-0.859% for FMOC-Leu and Pyridoxine HCl, respectively. (see supporting information Table S3) . The inter-day precision ST 3shows the percentage RSD 0.616% to 1.540%, and 0.664% to 1.008% where, three replicates of three concentrations of the standard solution of both the drugs were analyzed.

3.4.5. Different Analyst

For UV-visible spectroscopy measurement of three concentrations was taken by two analysts to confirm the precision of the method. The % RSD found were 0.256-0.84% and 0.790-1.499% for FMOC-Leu and Pyridoxine HCl respectively. Similarly for RP-HPLC, the % RSD was obtained in the range of 0.217507% to 1.245125% and 0.542433% to 1.36009% for FMOC-Leu and Pyridoxine HCl respectively. The results are tabulated in Table S4 (see supporting information) .Hence it could be said that the methods provide satisfactory results.

3.4.6. Different Instrument

The data generated using a different instrument for validation of FMOC-Leu and Pyridoxine HCl, the %RSD for a different instrument was found to be 0.209% to 1.215% and 0.970% to 1.201% respectively. The results are tabulated in Table S5 (see supporting information).

3.4.7. Robustness

For UV-visible spectroscopy, the robustness was determined by changing wavelength ±1nm. The % RSD for FMOC-Leu and Pyridoxine HCl was found to be 0.3549% to 1.778% and 0.794% to 1.035% respectively, while for RP-HPLC, robustness was validated by changing wavelength and changing the flow rate. The change in wavelength shows % RSD in the range of 0.66139% to 1.48447% and 0.04877% to 0.6099% while the change in flow rate by ± 0.2 mL/min shows % RSD of 0.661% to 1.006% and 0.0487% to 0.609%for FMOC-Leu and Pyridoxine HCl respectively. The results are tabulated in Table 5.

Table 5. Robustness showing change in wavelength (UV spectroscopy and HPLC) and change in flow rate (HPLC) for Pyridoxine HCl and FMOC-Leu

Change in Wavelength					
UV Spectroscopy					
Drug	**Conc. (µg/mL)**	**Wavelength 250nm**	**Wavelength 251nm**	**Wavelength 252nm**	**%RSD**
FMOC-Leu	29.3	0.05751	0.05886	0.05707	1.613
	43.9	0.08779	0.08973	0.08805	1.778
	58.6	0.11072	0.11134	0.11089	0.3549
Pyridoxine HCl	4	-0.01376	-0.01398	-0.01398	0.794
	6	-0.01778	-0.01781	-0.01753	0.868
	8	-0.02188	-0.0219	-0.0215	1.035
HPLC					
Drug	**Conc. (µg/mL)**	**Wavelength 284nm**	**Wavelength 285nm**	**Wavelength 286nm**	**%RSD**
FMOC-Leu	4	209130	210142	215001	1.48447
	6	261405	263418	264872	0.66139
	8	308273	312776	315235	1.13127
Pyridoxine HCl	29.3	516550	516574	516998	0.04877
	43.6	643610	645621	648721	0.3985
	58.6	759254	763263	768542	0.60999
Change in Flow rate					
Drug	**Conc. (µg/mL)**	**0.8 mL/min**	**1.0mL/min**	**1.2mL/min**	**%RSD**
FMOC-Leu	4	211746	210142	208895	0.679
	6	261405	264118	264872	0.661
	8	318255	311276	318335	1.006
Pyridoxine HCl	29.3	516550	516574	516998	0.0487
	43.6	643610	645621	648721	0.398
	58.6	759254	763263	768542	0.609

3.4.8. Accuracy

In the determination of the accuracy of the proposed method, UV-visible spectroscopy, and RP-HPLC, the known drug concentrations have been spiked in placebo at 50 %, 100 % & 150 %, and studies were carried out in triplicates for each level. Accuracy was determined based on the percentage of the recovery. The tabulated results are in Table S6 (see supporting information).

3.4.9. Limit of Detection (LOD) and Limit of Quantification (LOQ)

The values for LOD and LOQ after calculation in UV-visible spectroscopy was found to be 0.252 and 0.762 for pyridoxine HCl and 0.380 µg/mL and 1.153 µg/ mL for FMOC-Leu respectively while for RP-HPLC the LOD and LOQ were observed 0.0486 µg/ mL and 0.147 µg/ mL for pyridoxine HCl and 2.334 µg/ mL and 7.073 µg/ mL for FMOC-Leu respectively. The results are tabulated in Table S7 (see supporting information).

3.4.10. Statistical Comparison: UV-Visible Spectroscopy and RP-HPLC Methods

The comparison between the two methods UV-visible spectroscopy and RP-HPLC are summarized in Table 6, where F cal< F critical hence there observed no significant difference between the two methods. Either method can be adopted for the estimation of pyridoxine HCl and FMOC-Leu.

Table 6. Statistical comparison: UV-spectroscopy and HPLC methods

Drug	Source of Variation	SS	D_f	MS	F cal	P-Value	F critical
Pyridoxine HCl	Between Groups	0.040833	1	0.040833	0.349501	0.567518	4.964603
	Within Groups	1.168333	10	0.116833			
	Total	1.209167	11				
FMOC-Leu	Between Groups	0.053333	1	0.053333	0.073093	0.792384	4.964603
	Within Groups	7.296667	10	0.729667			
	Total	7.35	11				

F-critical obtained from the F-Distribution Table for 5% significant level and degree of freedom n1 = 1 (numerator) and n2 = 10 (denominator) was 4.96. Here F cal < F critical hence no significant difference was found for developed two methods

In conclusion, Selected methods for UV-visible spectroscopy and RP-HPLC [12-14] were reported for the estimation of Pyridoxine HCl and FMOC-Leu individually. The present method reports the use of dervatizing agent and the active hydrogen of a free amino group at Leucine was only replaced by non-polar group using N-(9-Fluorenylmethoxycarbonyloxy) succinimide (FMOC-OSU) as a derivatizing agent, resulting in conversion of Leucine to FMOC-Leucine derivative to make an analyte less reactive, volatile, and thus improve its chromatographic behavior. The pyridoxine HCl and FMOC-Leu were eluted at 3.4 and 14.3 min with a run time of 10 min. The current method was developed using 0.1% OPA with ACN: water in the ratio of (50:50% v/v). The developed method was found to be cost-effective, efficient, and sensitive with the reduced ratio of organic solvent in the mobile phase. The validation parameters are summarized in Table 4 suggesting the method was successfully validated according to ICH guidelines for all the parameters which were found within acceptance criteria.

The present study reports a unique and novel analytical method for analysis of Pyridoxine HCl and FMOC-Leu for which to date no analytical method has been reported. RP-HPLC was found to be powerful, yielded a well-resolved peak of the drug and a specific approach for quantification of Pyridoxine HCl and FMOC-Leu. The developed derivatized UV-visible spectroscopy method is simple, specific and reproducible, and precise. The method was validated to establish compliance in accordance with ICH guidelines. Statistical parameters and system suitability parameters were found to be acceptance criteria.

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Supporting Information:

Supporting information accompanies this paper on http://www.acgpubs.org/journal/ journal-of-chemical-metrology

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