

Stability Indicating Assay of Yohimbine Hydrochloride Using High Performance Liquid Chromatography

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Abstract: A sensitive method was established for the analysis of yohimbine (YOH) using high performance liquid chromatography (HPLC) with fluorescence detection. The proposed method is stability indicating for determination of yohimbine in presence of its degradation products, obtained by stressing yohimbine by extremes of pH in presence of heat. The assay method was proved to be sensitive and precise, where the precision of the assay was evaluated using the standard deviation or the mean coefficient of variation which were found to be within the acceptable limit of 2% and were specifically 0.303 & 0.358 % for intraday and interday samples respectively. The results showed that there is no interference between yohimbine and its degradation products since the retention time of yohimbine was found to be 6.1 minutes & that of its acid and alkaline hydrolysis induced degradation products is 4.12 minutes employing a mobile phase composed of 0.5% aqueous triethyl amine pH 3.0 " adjusted with orthophosphoric acid " and methanol in a ratio (65 : 35 v/v).

Key words: Yohimbine; Yohimbex; HPLC

INTRODUCTION

Yohimbine is the most important of the complex indole alkaloid types. It is derived from yohimbe bark which occurs in various tropical trees such as *Pausinystalia yohimbe*. Pierre, *Aspidos-perma quebrachoblanco* schiecht and as a minor Alkaloid in some *Rawaolfia* Species^[1]. Yohimbine is an indole alkaloid available commercially as yohimbex^R tablet. It's commonly used as an aphrodisiac and in treatment of male impotence^[2,9]. pharmacologically classified as an alpha-2 adrenoreceptor antagonist with some antidopaminergic properties^[10,13]. It can be also used for general purposes such as treatment of orthostatic hypotension^[14], and some forms of obesity^[15], diabetic neuropathy^[16].

Several methods have been reported for the analysis of yohimbine in pure form and pharmaceutical preparations such as spectrophotometric methods using modified vierordt method^[17], colourimetric methods^[18,19], and also electrochemical methods using anodic voltammetry^[20], potentiometric determination using picrate ion selective electrode^[21].

A few HPLC methods for determination of yohimbine in pharmaceutical preparations and biological samples using different techniques of detection as chemiluminescent^[22,23], coulometric^[24], amperometric, ultraviolet detection^[25,26], were discussed.

Another HPLC method was suggested for the analysis of yohimbine using 95% methanol as mobile phase and fluorescence detection^[27], which depends on

the native fluorescence of yohimbine (280 nm excitation, 360 nm emission) which has been rarely exploited in the development of a sensitive assay of this compound.

In this paper the main objective of the study was to develop a sensitive stability indicating assay of the parent compound (YOH) without interference from its degradation products. The method employs a mobile phase of 0.5% aqueous triethyl amine adjusted at pH (3.0) and methanol in a ratio of (65 : 35) % v/v and a flow rate of 1 ml/min using fluorescence detection (280 nm excitation, 360 nm emission).

Experimental:

Materials and Reagents: All Reagents were USP grade chemicals and HPLC grade methanol was obtained from sigma (Egypt); yohimbine hydrochloride 99.9% was kindly supplied by Amriya pharmaceuticals company (Alexandria, Egypt). yohimbex^R tablets: manufactured by Amriya pharmaceuticals company B.N.910508, each tablet was labeled to contain 5.4mg of yohimbine hydrochloride.

Instrumentation: An HPLC unit equipped with 20µl loop injector, and a spectro-fluorometer (Model RF-530, Shimadzu, Kyoto, Japan) excitation 280 nm, emission 360 nm. The chromatographic column from Agilent technologies, USA was bondapak C18 column 4.6x150 mm with a particle size of 5 µm. Data acquisition was performed on an Agilent LC chemstation software.

Standard Solutions:

Standard Stock Solutions:

Standard Stock Solution for Yohimbine Hydrochloride Solution: A portion equivalent to 10.0 mg of yohimbine hydrochloride (YOH.HCl) is transferred from the standard stock solution into 100 ml volumetric flask and the volume is made up to the mark with distilled water to give a standard working solution of 100.0 mg/ml-1.

Standard Stock Solution of Yohimbine Hydrochloride Degradates:

Standard Alkaline Degradate: It was prepared by mixing 10.0 mg of yohimbine HCl with 1.0 M NaOH, followed by heating in a boiling water bath at 100°C for 2 hours, the solution is then cooled and neutralized with a calculated volume of 1.0 M HCl and the volume is made up to the mark in a 100 ml volumetric flask with water. Complete degradation was checked by TLC using silica gel 60 F254 plates and a developing solvent system of methylene chloride, methanol and ammonium hydroxide in a ratio of (90:14:1).

Standard Acid Degradate: It was prepared by mixing a portion equivalent to 10.0 mg of yohimbine HCl with 10 ml 1.0 M HCl, followed by heating in a boiling water bath at 100°C for 2 hours, the solution is then cooled and neutralized with a calculated volume of 1.0 M NaOH and the volume is made up to the mark in a 100 ml volumetric flask with water. Complete degradation was checked by TLC using silica gel 60 F254 plates and a developing solvent system of methylene chloride, methanol, and ammonium hydroxide in a ratio of (90:14:1).

Standard working solutions:

Standard Working Solution for Yohimbine Hydrochloride: Five ml of the standard stock solution of yohimbine hydrochloride (100.0 µg/ml-1) is further diluted to 100.0 ml with the same solvent in a 100 ml volumetric flask. Then the diluted solution is used as a working standard solution of yohimbine hydrochloride of concentration 5000 ng/ml-1.

Standard Working Solution for Yohimbine Hydrochloride Degradates: Five ml of each of the standard stock solutions of the YOH.HCl degradates is further diluted to 100 ml with the same solvent in three 100 ml volumetric flask. Then the diluted solutions are used as a working standard solutions of the degradates of concentration 5000ng/ml-1.

Procedure:

Chromatographic conditions: Isocratic elution technique was utilized with the column that was

maintained at room temperature. The mobile phase used was a mixture of 0.5% aqueous triethylamine adjusted at PH 3.0 using orthophosphoric acid and methanol in a ratio of (65: 35 V/V). The mobile phase was filtered through a 0.45 µm membrane filtration system (Millipore Corp., Milford, MA, USA) to remove any particulate matter then degassed by sonication for 20 minutes. The flow rate was 1 ml/min. samples of 20µl were injected onto the column and the detector was set at excitation 280nm, emission 360 nm).

* All the chromatographic determinations are performed 5 times at ambient temperature.

Method Validation:

Linearity: Different aliquots (0.5-10.0 ml) of yohimbine hydrochloride from its working standard solution (5000 ng/ml-1) separately into 50 ml volumetric flasks, diluted to the volume with mobile phase to give concentrations of (50-1000ng/ml-1).

Each of those dilutions are then chromatographed by injecting an aliquot of 20 µl of each into the chromatographic system in this work. The mean peak areas of five determinations of each concentration are plotted against the corresponding concentrations and the regression equations are then computed.

Accuracy: Apply the previously mentioned procedure under linearity (2.4.2.1.) for different concentrations of YOH.HCl. Calculate the concentrations of the studied drug from its corresponding regression equations then calculate the mean percentage recoveries and standard deviations.

Precision:

Repeatability (Intraday Precision): The previously mentioned procedure under linearity (2.4.2.1.) is used for the analysis of three samples of concentrations (100.0, 600.0, 1000.0 ng/ml-1 for the determination of interday (n=5) and the relative standard deviations (RSD%) are calculated.

Intermediate Precision (Interday Precision): The previously mentioned procedure under linearity (2.4.2.1.) is repeated for the analysis of three samples of concentrations (100.0, 600.0, 1000.0 ng/ml-1) for the determination of interday (n=5) and the the relative standard deviations (RSD%) are calculated.

Selectivity: Six laboratory prepared mixtures containing a fixed amount of yohimbine hydrochloride and different ratios of each of its degradation products are prepared and chromatographed by adopting the procedure mentioned under linearity (2.4.2.1.). The concentrations were determined referring to the regression equation and the percentage recoveries are calculated.

Application to Pharmaceutical Preparation: Twenty tablets were accurately weighed, and finely ground in a mortar. A portion of the powder equivalent to 10.0 mg of yohimbine HCl was accurately weighed, extracted with a minimum amount of water, sonicated for 15 minutes, centrifuged for 10 minutes, the precipitate is then filtered through a micropore filter, washed into a 100 ml volumetric flask and the volume is made up to the mark with the same solvent to provide a solution of concentration 100.0 μgml^{-1} then further dilution is done using the mobile phase to obtain an aliquot equivalent to (100 ngml^{-1}). This aliquot is then chromatographed by adopting the procedures mentioned under linearity (2.4.2.1.), the concentration of the pharmaceutical preparation is calculated from the corresponding regression equation, the mean percentage recovery is then calculated.

Validation by Standard Addition Technique: This study was performed by adding known amounts of YOH.HCl from its working standard solution to a known concentration of the commercial preparation. The resulting mixtures are chromatographed by adopting the procedures mentioned under linearity (2.4.2.1.). The concentrations are determined referring to the regression equation and percentage recoveries are calculated.

RESULTS AND DISCUSSION

A validated, selective and sensitive HPLC method was proposed for the analysis of YOH. This method depends on the native fluorescence of yohimbine (excitation at 280 nm, emission at 360 nm) that has been rarely exploited in the development of a sensitive assay of this compound. The proposed method proved to be stability indicating since it was applied for the determination of the intact drug in presence of its degradates. The method also proved to be sensitive for the determination of YOH in the nanogram range.

Best separation was obtained using a bondapak C18 (150 mm X4.6 mm, i.d.) with a particle size of 5 μm analytical column and a mobile phase composed of a mixture of 0.5% aqueous triethyl amine adjusted at PH3.0 using orthophosphoric acid and methanol in a ratio of 65: 35% (v/v).

Under the previously mentioned chromatographic conditions, yohimbine was found to show a peak at a retention time of 6.10 ± 0.094 minutes, whilst the acid and the alkaline degradates showed a peak at 4.123 ± 0.005 minutes which belongs to yohimbic acid⁽²⁹⁾ which is a common degradation product in both acid and alkaline hydrolysis as shown in figures (2&3) with the exception that the alkaline degradate showed another peak of the harman degradation product ($\text{C}_{12}\text{H}_{10}\text{N}_2$) which is a minor degradation

product in alkaline hydrolysis⁽³⁰⁾ which appears at 4.70 ± 0.014 minutes and it only appears at relatively high concentrations of the alkaline degradate (in micrograms) as shown in figure(4).

It was observed by using a methanol concentration of 35%, the retention time of YOH was 6.10 ± 0.0869 minutes as shown in figure (1), thus the resolution factor (Rs) was found to be 5.6 and thus excellent separation was achieved between the drug and its degradates.

The mean peak areas of five determinations of each concentration is plotted against its corresponding concentration in order to obtain the calibration graph.

The proposed method was found to be linear over the range of (50-1000 ngml^{-1}) as shown in figure (5). The linear regression equation was computed and found to be:

$$\text{P.A} = 0.369 \text{ C} + 3.6568 \quad r = 1$$

Where P.A is the mean peak area of five determinations, C is the concentration of YOH in ngml^{-1} , and r is the correlation coefficient.

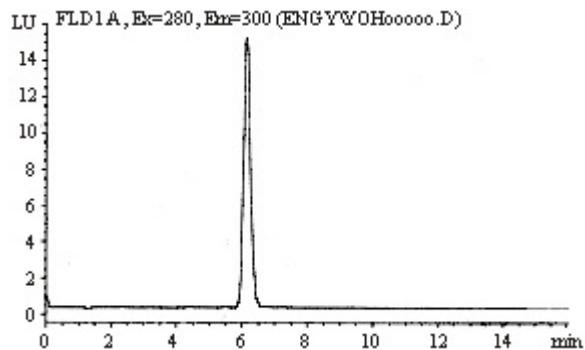


Fig. 1: Scanning profile HPLC chromatogram of 600.0 ngml^{-1} of yohimbine hydrochloride using fluorescence detection

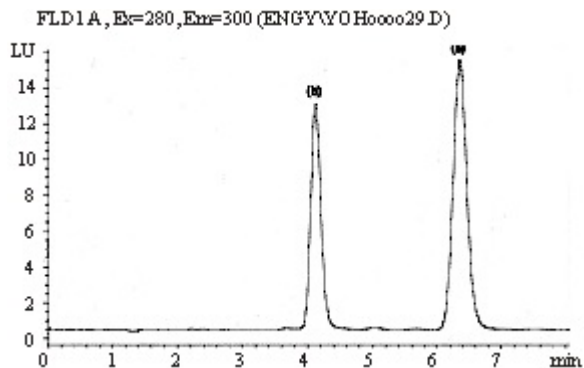


Fig. 2: Scanning profile of HPLC chromatogram of yohimbine hydrochloride (a) and its alkaline degradate (b) each of (600.0 ngml^{-1}) using fluorescence detection.

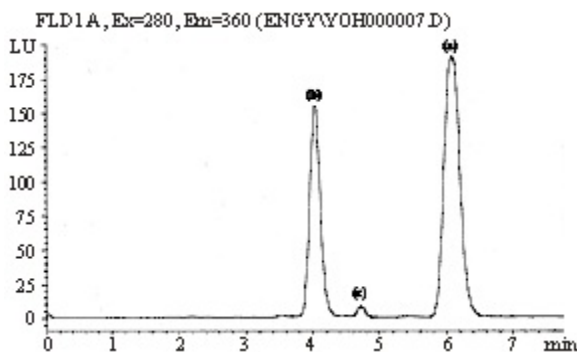


Fig. 3: Scanning profile of HPLC chromatogram of yohimbine hydrochloride (a) and its alkaline degradate (b,c) each of (10.0µgml⁻¹) using fluorescence detection.

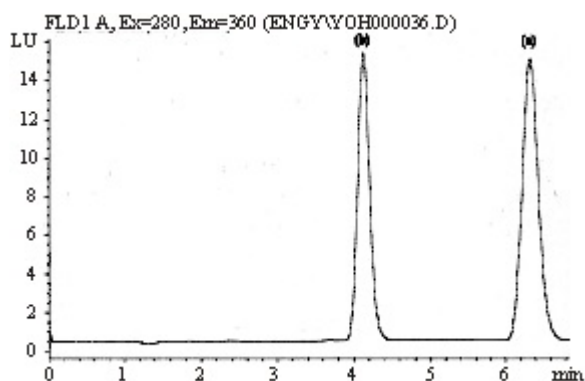


Fig. 4: Scanning profile of HPLC chromatogram of yohimbine hydrochloride (a) and its acid degradate (b) each of (600.0 ngml⁻¹) using fluorescence detection

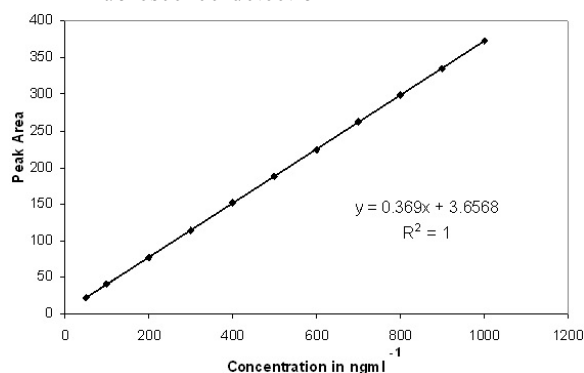


Fig. 5: Linearity of peak area versus concentration of yohimbine hydrochloride in ngml⁻¹ by high performance liquid chromatography.

The validity of the proposed method was assessed by the determination of YOH in pure form. The values of the percentage recoveries are listed in table (1).

The stability indicating nature of the assay was confirmed by analyzing several laboratory prepared mixtures of YOH and its degradates which reproduced different composition ratios. The results compiled in tables (2&3) were found satisfactory which is evident from the mean percentage recoveries and standard deviations.

Table 1: Accuracy of the proposed high performance liquid chromatographic method for the analysis of pure samples of yohimbine hydrochloride using fluorescence detection.

Taken (µgml ⁻¹)	Found* (ngml ⁻¹)	Recovery %
50.00	50.33	100.65
100.00	99.47	99.47
200.00	200.22	100.11
300.00	300.00	100.00
400.00	400.00	100.00
600.00	599.99	100.00
800.00	800.00	100.00
1000.00	1000.00	100.00
Mean±SD		100.03 ±0.318

* Average of five determinations.

The specificity of the method was investigated by observing any interference encountered from the excipients during the analysis of tablets. It was shown that these additives do not interfere with the proposed method. The accuracy of the proposed method was further confirmed by applying the standard addition technique. The percentage recoveries of the authentic added are demonstrated in table (4). The excellent recoveries of the standard addition method suggest the good accuracy of the proposed method. Table (5) shows the results of statistical analysis of experimental data such as slopes, intercepts, correlation coefficient obtained by the linear least square treatment of the results along with standard deviation of the slope (Sb), intercept (Sa), on the ordinate. The good linearity of the calibration graphs and the negligible scatter of the experimental points are clearly evident by the values of the correlation coefficients and standard deviations. The table also shows also the detection limit and the quantitation limit of the drug.

System suitability criteria including retention time (minutes), capacity factor(k'),selectivity factor, resolution (Rs), , number of theoretical plates (N), and the symmetry are displayed in table (6).

Statistical analysis of the results using the t-test and F-value indicating that there is no significant difference between the suggested method and the official HPLC method regarding accuracy and precision are shown in table (7).

Table 2: Results obtained for the analysis of laboratory prepared mixtures containing different ratios of yohimbine and its alkaline degradate by the proposed high performance liquid chromatographic method using fluorescence detection .

Sample No.	Intact drug (ngml ⁻¹)	Alkaline degradate (ngml ⁻¹)	Recovery %*
1	600.00	100.00	99.62
2	600.00	200.00	100.32
3	600.00	300.00	100.28
4	600.00	400.00	100.25
5	600.00	500.00	98.99
6	600.00	600.00	100.18
Mean±SD			99.94±0.533

* Average of five determinations.

Table (3): Results obtained for the analysis of laboratory prepared mixtures containing different ratios of yohimbine and its alkaline degradate by the proposed high performance liquid chromatographic method using fluorescence detection .

Sample No.	Intact drug (ngml ⁻¹)	Alkaline degradate (ngml ⁻¹)	Recovery %*
1	600.00	100.00	99.59
2	600.00	200.00	99.33
3	600.00	300.00	99.50
4	600.00	400.00	100.15
5	600.00	500.00	98.91
6	600.00	600.00	98.99
Mean±SD			99.41±0.452

* Average of five determinations.

Table 4: Quantitative determination of yohimbine hydrochloride in pharmaceutical preparation and application of standard addition technique by the proposed high performance liquid chromatographic method using fluorescence detection.

Pharmaceutical preparation	Taken (ngml ⁻¹)	Found % * ±SD	Standard addition technique		
			Pure added (µgml ⁻¹)	Pure found* (µgml ⁻¹)	Recovery %
Yohimbex® tablets claimed to contain 5.4 mg YOH.HCl/tablet (Batch No. 910508)	100.00	99.26±0.645	100.00	99.15	99.15
			300.00	297.30	99.10
			500.00	492.36	98.47
			700.00	690.55	98.65
			900.00	894.50	99.39
Mean±SD					98.95±0.380

* Average of five determinations.

Table 5: Assay validation parameters of the proposed high performance liquid chromatographic method using fluorescence detection for the determination of pure yohimbine hydrochloride.

Parameters	HPLC method
Range	50-1000 µg ml ⁻¹
Linearity	
Slope	0.369
Intercept	3.6568
Correlation coefficient (r)	1
Standard error of the slope	7.89x10 ⁻⁵
Confidence limit of the slope	0.368799-0.369156
Standard error of the intercept	0.046702
Confidence limit of the intercept	3.551171-3.762466
Standard error of estimation	0.080952

Accuracy (mean±SD)	100.03±0.318
Precision(RSD%)	
Repeatability*	
Intermediate precision*	0.303
	0.358
LOD**	0.26 ngml ⁻¹
LOQ**	0.80 ngml ⁻¹

Table 6: The system suitability test results of developed HPLC fluorescence method for the determination of yohimbine hydrochloride in presence of its degradates.

System suitability parameters of YOH.HCl in presence of:	Retention time (min)	Capacity factor (K')	Selectivity (α)	Number of theoretical plates (N)	Resolution (Rs)	Symmetry
In presence of acid and alkaline degradates	6.10±0.0869	1.62	2.48	4205	6.55	0.83

Table 7: Statistical comparison of the results obtained by applying the proposed high performance liquid chromatographic method and the official method for the determination of yohimbine hydrochloride in pharmaceutical preparation.

Items	HPLC method	Official method ⁽¹⁷⁰⁾
Mean	99.26	100.08
SD	0.645	1.203
RSD%	0.650	1.202
N	5	5
Variance	0.416	
Student's t-test (2.160)	1.347	
F-value (3.633)	3.476	

* The values between parenthesis are the theoretical values of t- & F- at P=0.05.

Conclusion: A simple, specific, and sensitive HPLC method has been developed using a single isocratic system for the determination of YOH in bulk material and in its dosage form. The mobile phase used is simple, since it contains no buffer system. No interference was encountered from the possible degradation products and therefore it can be used as a stability indicating method. The method was found to be more sensitive than HPLC coupled with UV detection since it can determine YOH in very small concentrations (ngml^{-1}).

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