Estimation of yohimbine base in complex mixtures by quantitative HPTLC application

Maged Saad Adel-Kader[1,3]*, Naif Wahebi Hamadan Alwahebi[2] and Prawez Alam[1]
[1]Department of Pharmacognosy, College of Pharmacy, Prince Sattam Bin Abdulaziz University, Al-Kharj, Saudi Arabia
[2]Undergraduate Student, College of Pharmacy, Prince Sattam Bin Abdulaziz University, Al-Kharj, Saudi Arabia
[3]Department of Pharmacognosy, College of Pharmacy, Alexandria University, Alexandria, Egypt

Abstract: The indole alkaloid Yohimbine has been used for over two centuries in the treatment of erectly dysfunction. Several formulations containing yohimbine salts, yohimbe bark power or extract are marketed worldwide. Determination of the amount of yohimbine in such formulation is a challenging task due to their complex nature. Extraction followed by acid-base purification resulted in a relatively pure alkaloids containing fractions. The exact amounts of yohimbine free base in different formulations were determined by densitometric HPTLC validated methods using silica gel TLC plates. Standard curve *for* yohimbine was generated using yohimbine hydrochloride subjected to the same acid-base treatment as the used samples. All formulations found to contain yohimbine though some with less concentration than the labeled amount.

Keywords: *Yohimbe bark*; yohimbine; quantitative; HPTLC.

INTRODUCTION

Pausinystalia johimbe, (Rubiaceae), known as Yohimbe, is a plant species native to western and central Africa (Nigeria, Cabinda, Cameroon, Congo-Brazzaville, Gabon, Equatorial Guinea) (Kew World Checklist). The total extract of Yohimbe bark contains about 6% indole alkaloids. Yohimbine (17α-hydroxyyohimban-16α-carboxylic acid methyl ester) represents 10-15% of the indole alkaloids content (Tam et al. 2001). Yohimbine is a pharmacologically well-characterized α_2-AR antagonist that has been used for over a century in the treatment of erectly dysfunction (Morales, 2000). Yohimbine improves impotence via increasing the penile blood flow as well as increasing central excitatory impulses to the genital tissue (Wagner and Saenz, 1998, Balon 1999). It is significantly more active at presynaptic adrenoreceptors than postsynaptic receptors. This action blocks the decrease in central noradrenergic response and blocks the reduction in peripheral sympathetic activity (Goldberg et al 1983, Anden et al, 1982, Charney 1982).

Yonimbine was estimated utilizing a fluorimetric method with other two alkaloids: serpentine and boldine (Gürkan, 1976). Stability-indicating simple HPLC method was developed for the analysis of yohimbine using RP column and water/methanol as mobile phase (Mittal et al, 2000). A method for extraction and capillary gas chromatographic (GC) separation of the alkaloid was developed to analyze a number of commercial yohimbe products (Betz, 1995). Quantitation of yohimbine was also accomplished by non-aqueous capillary electrophoresis with diode array detection (Chen et al, 2008).

**Corresponding author:* e-mail: mpharm101@hotmail.com

Twenty-six commercial yohimbe products were subjected to chemical analysis. Most of the analyzed samples were free yohimbine (Betz, 1995). Yohimbine HCl was estimated in five formulations containing yohimbine HCl or Yohimbe bark powder by validated HPTLC method (Badr, 2013). Many marketed pharmaceutical formulations are present in the local market containing Yohimbe bark powder or bark extract in addition to other substances in the form of hard or soft gelatin capsules or tablets. Detection and estimation of yohimbine in such complex mixture represents a real challenge especially when present in minute concentrations.

MATERIALS AND METHODS

Standards and chemicals

Standard Yohimbine Hydrochloride (fig. 1) was obtained from Sigma-Aldrich, St. Louis, MO, USA. HPLC grade solvents and analytical reagents (AR) were used. TLC plates 10 × 20 cm glass-backed plates coated with 0.2 mm layers of silica gel 60 F254 (E-Merck, Germany) were used for the analyses.

Pharmaceutical preparations

Four marketed pharmaceutical preparations containing either yohimbine salts, Yohimbe bark extract or Yohimbe bark powder were purchase from Pharmacies in Riyadh, KSA and Alexandria, Egypt and used for analysis. Sample A: Soft gelatin capsules containing 200mg Ginkgo biloba extract, 700mg Royal jelly, 500mg Siberian Ginseng extract, 300mg Saw palmetto extract, 200mg Yohimbe bark powder and 15mg Zinc; Sample B: Hard gelatin capsules containing 451mg Yohimbe bark extract; Sample C: Hard gelatin capsules containing 5.4mg yohimbine HCl, 15mg Vitamin E, 25mg Caffeine and 5mg Nicotinic acid; Sample D: tablets containing

450mg Yohimbe bark extract, 450mg Siberian Ginseng extract and 30 IU Vitamin E.

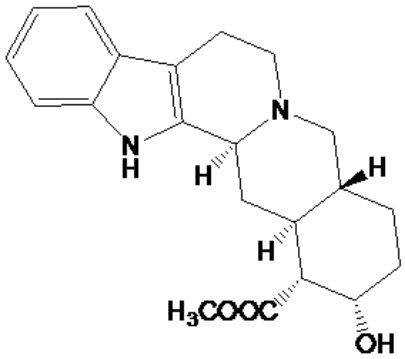

Fig.1: Chemical structure of yohimbine.

Sample preparation for analysis of yohimbine in Pharmaceutical preparations

Soft gelatin capsules were cut using cutter and the dark viscous contents of 5 capsules were extracted with methanol. Contents of 10 hard gelatin capsules were extracted with 25mL methanol, filtered and the process was repeated 3 times. Five tablets were crushed and extracted with methanol 3 times as above. The methanol extracts from each sample were separately diluted with water and rendered alkaline with ammonium hydroxide. The aqueous layers were extracted with $CHCl_3$ (5 X 300mL). The combine $CHCl_3$ layers were separately evaporated, dissolved in 3:1 acidulated water/methanol and extracted with *n*-hexane (3 X 200mL). The *n*-hexane layers were rejected and the aqueous layers were again rendered alkaline with ammonium hydroxide, extracted with $CHCl_3$ (5 X 300mL). The combine $CHCl_3$ layers were evaporated and dissolved in $CHCl_3$, completed to volumes in volumetric flasks and kept in refrigerator till time of application on TLC.

Chromatographic conditions

HPTLC densitometric analysis was performed using 10 × 20cm glass-backed plates coated with 0.2mm layers of silica gel 60 F254. Samples application as 6mm bands on the TLC plates were performed using Camag Automatic TLC Sampler 4 (ATS4) sample applicator (Switzerland) equipped with Camag micro litre syringe. The application was fixed at the rate of 150nl/s. Concentrations of samples were adjusted to be included within the range of the used concentrations of standard yohimbine (200- 1000 ng/spot) as expected from the labeled amounts in each sample. The TLC plates were developed in Camag Automatic Developing Chamber 2 (ADC2) previously saturated with mobile phase vapour for 30 min at 22°C. Mobile phase composition was $CHCl_3$-MeOH-ammonia 90:9.5:0.5 (%, *v/v*) developed by linear ascending technique to the distance of 80 mm. Camag TLC scanner in absorbance mode was used to scan the TLC plates at 221 nm using the deuterium lamp (figs. 2, 3). Scanning conditions were fixed at 20 mm/s scan speed and 4.00 × 0.45 mm slit dimensions.

Calibration curve of yohimbine by HPTLC method

Accurately weighed 100mg of authentic yohimbine hydrochloride (98% pure) was dissolved in methanol, diluted with water and rendered alkaline with ammonium hydroxide. The aqueous layer was extracted with $CHCl_3$ (5 X 300mL). The combine $CHCl_3$ was evaporated and residue was dissolved in $CHCl_3$ in a 100mL volumetric flask. From this solution 1mL was further diluted with methanol in a 10ml volumetric flask to get concentration of 100µg/mL. The obtained solution was used as a stock solution for standard yohimbine. Different volumes of working standard, i.e. 2, 4, 6, 8 and 10µL were applied on TLC. The calibration curve was plotted between the two parameters; peak areas and concentration per spot in the range of 200-1000ng/spot (fig. 4).

Method validation

Linearity

Peak area obtained from application of 200-1000ng/spot to prove the linearity of the method. Linearity data was statistically treated using least square linear regression analysis (table 1).

Accuracy

Accuracy was determined by standard addition method. Sample of yohimbine containing (200ng/spot) was spiked after analysis with the extra 0, 50, 100 and 150% of the standard yohimbine and the solutions were analyzed one more time in six replicates by the developed method. The % recovery and % relative standard deviation (% RSD) were calculated at each concentration level (table 2).

Precision

Repeatability and intermediate precision were determined to prove the precision of the method. Repeatability was determined as intraday precision whereas intermediate precision was determined by carrying out inter-day variation for the determination of yohimbine at three different concentration levels of 300, 400 and 500ng/spot in six replicates (table 3).

Robustness

Robustness of the proposed HPTLC methods was tested to evaluate the influence of small-intended changes in the chromatographic conditions during the quantification. Robustness was determined by inducing slight changes in the polarity of the mobile phase (table 4).

Limit of detection and quantification

Limit of detection (LOD) and limit of quantification (LOQ) were obtained by standard deviation (SD) method. They were calculated from the slope of the calibration (S) curve and SD of the blank sample using the equations:

$LOD = 3.3\times SD / S$

$LOQ = 10\times SD / S$

Table 1: Linear regression data for the calibration curve of Yohimbine (n=6).

Linearity range (ng/spot)	200-1000
Regression equation	Y = 6.682x + 297.12
Correlation coefficient	0.9973
Slope ± SD	6.682± 0.3948
Intercept ± SD	297.12 ± 270.39
Standard error of slope	0.2280
Standard error of intercept	156.12
95% confidence interval of slope	8.303- 9.319
95% confidence interval of intercept	805.83- 1501.5

Table 2: Accuracy of the proposed method (n=6).

Excess drug added to analyte (%)	Theoretical Content (ng)	Conc. Found (ng) ± SD	% Recovery	% RSD
0	200	194.17 ± 1.94	98.08	0.99
50	300	296.83 ± 2.64	98.94	0.89
100	400	393.83 ± 5.91	98.46	1.50
150	500	495.17 ± 4.02	99.03	0.81

Table 3: Precision of the proposed method

Conc. (ng/spot)	Repeatability (Intraday precision)			Intermediate precision (Interday)		
	Area ± SD (n = 6)	Standard error	% RSD	Area ± SD (n = 6)	Standard error	% RSD
300	3032±50	20.29	1.64	3024±57	23.47	1.90
400	4429±59	24.03	1.33	4434±68	27.89	0.52
500	5485±37	15.18	0.68	5488±41	16.60	0.74

Table 4: Robustness of the proposed method

Conc. (ng/spot)	Mobile phase composition (chloroform: methanol)			Results		
	Original	Used		Area ± SD (n = 6)	% RSD	R_f
		9.1:0.9	-0.1	4430±63	1.42	0.39
600	9.2:0.8	9.8:0.8	0.0	4436±56	1.26	0.38
		9.3:0.7	+0.1	4420±54	1.21	0.37

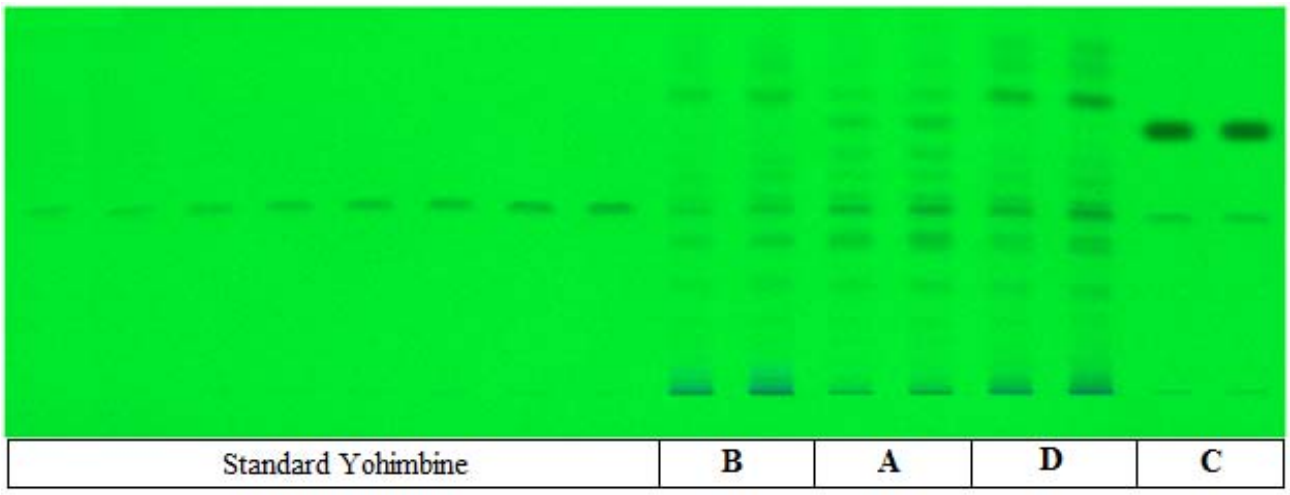

Fig. 2: TLC chromatogram of standard yohimbine and marketed formulations A-D using $CHCl_3$-MeOH-ammonia 90:9.5:0.5 (%, *v/v*) as developing system visualized under UV light.

Table 5: Yohimbine concentration in different formulations

Code	mg/Unite	Labeled amount/Unite
A	1.2	200 mg Yohimbe Bark powder
B	12.48	451 mg Yohimbe Bark extract ≡ 9mg Yohimbine
C	3.24	5.4 mg Yohimbine
D	3.75	450 mg Yohimbe Bark powder

Specificity

Spots in the analyzed samples with the same R_f values and spectra as that of yohimbine standards proved that the method is specific and exclude any interference from extraneous materials.

Quantification of yohimbine in pharmaceutical formulations

The test sample solutions were applied on the TLC, developed and scanned under the same conditions as for analysis of standard yohimbine. The peak areas of the spots with same R_f value as authentic were measured and the concentrations were calculated from the regression equation obtained from the calibration plot.

RESULTS

Method validation

The guidelines of international conference on harmonization (ICH guidelines 1996) were followed to validate the proposed HPTLC method. The results of linearity is presented in table 1. The obtained regression equation was Y=4.057X + 1659.395 with correlation coefficients (R^2) of 0.99312. Assurance of method accuracy was proved by % recovery and % RSD as presented in table 2. Precision of the method was proved by calculation of RSD for repeatability and intermediate precision (table 3). After introducing small changes into the densitometric TLC procedure the % RSD (table 4) was calculated to prove the robustness of the proposed method. LOD and LOQ of the proposed methods were found to be 23.58 and 71.44ng respectively. Estimation of yohimbine contents in some formulations are presented in table 5.

DISCUSSION

Sample preparation for analysis of yohimbine in Pharmaceutical preparations

Products available in the market contain either salts of yohimbine, Yohimbe bark extract or Yohimbe bark powder. Yohimbine is present in the form of salts with different anions or as freebase in the last two forms. Moreover, many of these formulations contain other plant extracts such as Ginkgo biloba extract, Royal jelly, Siberian Ginseng extract and Saw palmetto extract. The multi components of such extracts expected to interfere with the chromatograms of yohimbine during the quantification. Some formulations such as Sample A contain minute amounts of yohimbine that will almost be impossible to detect in the presence of other components. All samples were extracted with methanol as a strong solvent for all ingredients. The methanol extract subjected to acid-base treatment for purification of yohinbine from other non-alkaloidal components. However, in acid medium purification was made with *n*-hexane to avoid any loss of yohimbine HCl in stronger organic solvent. The alkaloids containing fraction was then estimated for yohimbine using yohimbine standard obtained by treating yohimbine HCl (*Sigma-Aldrich*) with the same acid-base procedures. The used method of extraction including the acid-base treatment allows complete extraction, converting yohimbine to free base and resulted in a well resolved chromatograms with no interferences from other spots. Such chromatogram resolution allows the detection of Yohimbine in the most complex sample A (fig. 2). The failure of detection of yohimbine in some formulation (Betz, 1995; Badr, 2013) may be due to improper extraction and purification steps.

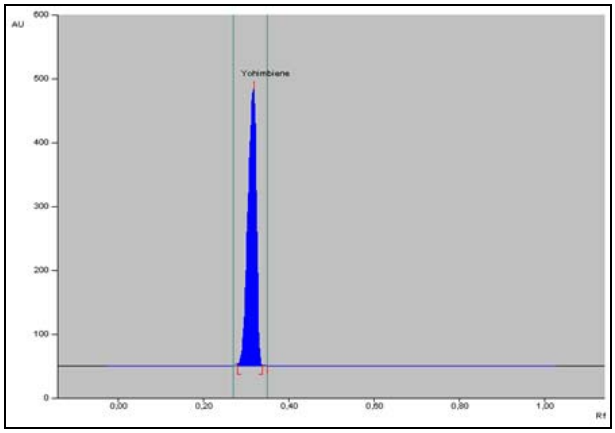

Fig. 3: HPTLC chromatogram of standard yohimbine spot measured at 221 nm.

Chromatographic conditions

Yohimbine showed 3 absorption bands, two maxima at 221, 281 and shoulder at 358 nm (fig. 5). The developing system was optimized in order to get uniform spots enable accurate TLC densitometric methods for analysis of yohimbine. The developing system composed of $CHCl_3$-MeOH-ammonia 90:9.5:0.5 (%, *v/v*) resulted in symmetrical and well resolved peaks with sharp apexes at R_f value of 0.38 (figs. 2, 3).

Method validation

The guidelines of international conference on harmonization (ICH guidelines 1996) were followed to validate the proposed HPTLC method.

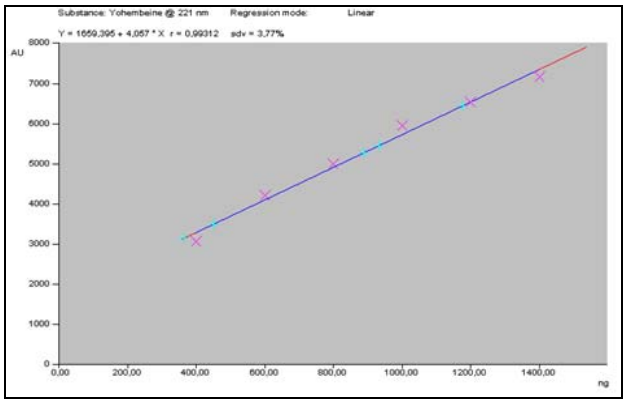

Fig. 4: Calibration curve of standard yohimbine using concentrations between 200-1200 ng/spot showing the linearity in the used rang.

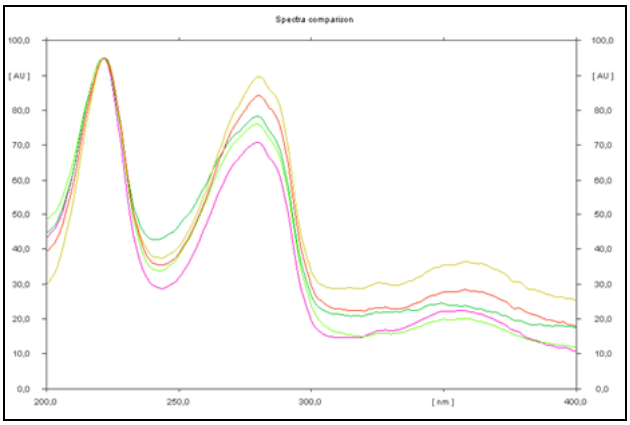

Fig. 5: UV spectra of yohimbine in the chromatograms of the standard and pharmaceutical formulations measured between 200-400 nm.

Linearity

The linearity range of the standard yohimbine covers the range between 200-1200 ng/spot (table 1). The obtained regression equation was Y=4.057X + 1659.395 with correlation coefficients (R^2) of 0.99312 (table 1).

Accuracy

Assurance of method accuracy was proved by recovery of 98.08-99.03% after spiking additional standard drug solution to the previously analyzed sample solutions. The low values of % RSD (0.81-1.50) (table 2) give a good indication for the accuracy of the current methods.

Precision

RSD was in the range 0.68- 1.64 for repeatability, 0.52-1.90 for intermediate precision (table 3). These values are mathematic reflection for method precision.

Robustness

After introducing small changes into the densitometric TLC procedure the % RSD values range was 1.21- 1.42 (table 4). These results proved the robustness of the proposed HPTLC method.

Limit of detection and quantification

LOD and LOQ of the proposed methods were found to be 23.58 and 71.44ng respectively. These values indicate that the method is suitable for the detection and quantification of minute amounts of yohimbine effectively even if present in complex matrix.

Specificity

All the analyzed samples showed spots with same R_f values and spectra as that of yohimbine standards. No any interference from extraneous materials disturbed the quantification. This resolution of spots proved that the method is specific (fig. 2).

Quantification of yohimbine in pharmaceutical formulations

The study showed that all formulations contain yohimbine as claimed. Both samples A and D contain Yohimbe bark powder and the estimated amounts of yohimbine were 0.6 and 0.8% in respectively. It is worth to mention that the % of yohimbine detected in sample A was 0.00063 demonstrating the ability of the method to detect minute amounts of yohimbine. Sample B showed 12.48 mg/capsule. However, in sample C the amounts of yohimbine were less than the labelled concentrations. Yohimbine contents estimated in formulations are presented in table 5.

CONCLUSION

The application of TLC densitometric method for quantitative analysis of yohimbine in complex formulations provides accurate, quick and easy tool for the analysis of many samples simultaneously. Other ingredients can be minimized in the chromatogram using acid-base extraction procedures efficient for obtaining relatively pure alkaloids containing fractions. Pretreatment allows the quantification of yohimbine in minute amounts in the corresponding samples. The application can expand to analyze other pharmaceutical formulations with different active ingredients.

ACKNOWLEDGEMENT

This research was supported by the Sheikh Abdu Allah bin Zaid bin Ghonaim Research Chair in Prophetic Medicine, Developing and Manufacturing Natural Products.

REFERENCES

Anden NE, Pauksens K and Svensson K (1982). Selective blockade of brain alpha 2-autoreceptors by yohimbine: effects on motor activity and on turnover of noradrenaline and dopamine. *J. Neural Transm.*, **55**: 111-120.

Badr JM (2013). A validated high performance thin layer chromatography method for determination of yohimbine hydrochloride in pharmaceutical preparations. *Pharmacogn. Mag*., **9**: 4-8.

Balon R (1999). The effects of anitdepressants on human sexuality: Diagnosis and management update. *Primary Psychology.,* **6**: 40-54.

Betz JM, White KD and der Marderosian AH (1995). Gas chromatographic determination of yohimbine in commercial yohimbe products. *J. AOAC. Int.,* **78**: 1189-1194.

Charney DS, Heninger GR and Sternberg DE (1982). Assessment of alpha 2 adrenergic autoreceptorfunction in humans: effects of oral yohimbine. *Life. Sci.,* **30**: 2033-2041.

Chen Q, Li P, Zhang Z, Li K, Liu J and Li Q (2008). Analysis of yohimbine alkaloid from *Pausinystaliayohimbe* by non-aqueous capillary electrophoresis and gas chromatography-mass spectrometry. *J. Sep. Sci.,* **31**: 2211-2218.

Goldberg MR and Robertson D (1983). Yohimbine: A pharmacological probe for study of the alpha 2-adrenoreceptor. *Pharmacol. Rev.,* **35**: 143-180.

Gürkan T (1976). Fluorimetric analysis of alkaloids. Determination of serpentine, yohimbine and boldine. *Microchimica Acta.,* **65**: 173-180

International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use: Harmonised Triplicate Guideline on Validation of Analytical Procedures: Methodology, Recommended for Adoption at Step 4 of the ICH Process on November 1996 by the ICH Steering Committee, IFPMA, Switzerland.

Kew World Checklist of Selected Plant Families, *Pausinystalia johimbe*, http://apps.kew.org/wcsp/namedetail.do?name_id=148191.

Mittal S, Alexander KS and Dollimore D (2000). A high-performance liquid chromatography assay for yohimbine HCl analysis. *Drug Dev. Ind. Pharm.,* **26**: 1059-1065.

Morales A (2000). Yohimbine in erectile dysfunction: The facts. *Int. J. Impot. Res.,* **12**: 70-74.

Tam S, Worcel M and Wyllie M (2001). Yohimbine: A Clinical Review. *Pharmacol & Ther.,* **91**: 215-243.

Wagner G and Saenz de TejadaI (1998). Update on male erectile dysfunction. *Br. Med. J.,* **316**: 678-682.