Estimation of yohimbine base in complex mixtures by quantitative HPTLC application

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Abstract: The indole alkaloid Yohimbine has been used for over two centuries in the treatment of erectile 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 α2-AR antagonist that has been used for over a century in the treatment of erectile 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). Yohimbine improves impotence via increasing central noradrenergic response and blocks the reduction in peripheral sympathetic activity (Goldberg et al 1983, Anden et al, 1982, Charney 1982).

Yohimbine 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).

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...
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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 CHCl₃ (5 X 300mL). The combine CHCl₃ was evaporated and residue was dissolved in CHCl₃ 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:

\[ \text{LOD} = 3.3 \times \text{SD} / S \]
\[ \text{LOQ} = 10 \times \text{SD} / S \]
**Table 1**: Linear regression data for the calibration curve of Yohimbine (n=6).

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

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

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

**Table 3**: Precision of the proposed method

<table>
<thead>
<tr>
<th>Conc. (ng/spot)</th>
<th>Repeatability (Intraday precision)</th>
<th>Intermediate precision (Interday)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Area ± SD (n = 6)</td>
<td>Standard error % RSD</td>
</tr>
<tr>
<td>300</td>
<td>3032±50</td>
<td>20.29 1.64</td>
</tr>
<tr>
<td>400</td>
<td>4429±59</td>
<td>24.03 1.33</td>
</tr>
<tr>
<td>500</td>
<td>5485±37</td>
<td>15.18 0.68</td>
</tr>
</tbody>
</table>

**Table 4**: Robustness of the proposed method

<table>
<thead>
<tr>
<th>Conc. (ng/spot)</th>
<th>Mobile phase composition (chloroform: methanol)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Original</td>
<td>Used</td>
</tr>
<tr>
<td>600</td>
<td>9.1:0.9</td>
<td>-0.1</td>
</tr>
<tr>
<td></td>
<td>9.8:0.8</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>9.3:0.7</td>
<td>+0.1</td>
</tr>
</tbody>
</table>

**Fig. 2**: TLC chromatogram of standard yohimbine and marketed formulations A-D using CHCl₃-MeOH-ammonia 90:9.5:0.5 (% v/v) as developing system visualized under UV light.
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 yohimbine 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.

Table 5: Yohimbine concentration in different formulations

<table>
<thead>
<tr>
<th>Code</th>
<th>mg/Unite</th>
<th>Labeled amount/Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.2</td>
<td>200 mg Yohimbe Bark powder</td>
</tr>
<tr>
<td>B</td>
<td>12.48</td>
<td>451 mg Yohimbe Bark extract = 9mg Yohimbine</td>
</tr>
<tr>
<td>C</td>
<td>3.24</td>
<td>5.4 mg Yohimbine</td>
</tr>
<tr>
<td>D</td>
<td>3.75</td>
<td>450 mg Yohimbe Bark powder</td>
</tr>
</tbody>
</table>

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.

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Estimation of yohimbine base in complex mixtures by quantitative HPTLC application


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