

VALIDATION OF HPLC METHOD WITH UV-DETECTION FOR DETERMINATION OF YOHIMBINE CONTAINING PRODUCTS

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Abstract: The aim of current investigation was the validation of HPLC method with UV-detection for identification and determination of Yohimbine by using column Spherisorb ODS RP C₁₈ (250 mm x 4.6 mm x 5 μm), temperature: 25 °C, mobile phase: methanol : acetonitrile : water = 70 : 20 : 10 v/v, flow rate: 2 ml/min., UV-detection: λ = 270 nm.

HPLC method was validated in accordance of linearity, limit of detection, limit of quantitation, accuracy, precision.

Linear dependence between concentration and peak area in range: 5.10⁻⁷ g/ml ÷ 1.10⁻⁵ g/ml was proved by regression equation: $y = 94517560831 \cdot x + 25119$. LOD = 5.44 10⁻⁷ g/ml; LOQ = 1.65.10⁻⁶ g/ml.

Accuracy was presented by the degree recovery R [%] ± RSD [%]: 98.68 % ± 2.03 %. For estimation of precision all data for Yohimbine content are included in confidence interval: 9.6 mg ÷ 10.14 mg.

Key words: HPLC, Yohimbine products (supplements, drugs).

Introduction

Yohimbine (Fig. 1.) is an indole alkaloid and was first isolated from bark of a West African tree *Pausinystalia yohimbe* (*K. Schumann*) *Pierre ex Beille* (*Pausinystalia yohimbe*, *Corynanthe yohimbe*, *Corynanthe yohimbi*, *Corynanthe yohimbi*) (*Rubiaceae*). The presence of Yohimbine has been reported in the bark of the following other plants: *Alchornea floribunda* Müll. Arg., *Aspidosperma quebrachoblanco* Schltdl., *Corynanthe paniculata* Welw, *Pausinystalia angolensis* Wernham, *Pseudocinchonia africans* Aug. Chev, *Rauwolfia nitida* Jacq., *Rauwolfia serpentina* (L.) Benth. ex Kurz., *Rauwolfia verticillata* (Lour.) Baill., *Rauwolfia vomitoria* Afzel [1].

Yohimbine is distributed in plants in tropical West Africa (Cameroon, Congo, Equatorial Guinea, Gabon, Nigeria). In West Africa yohimbine bark preparations were traditionally used as a general tonic, as a performance enhancer for

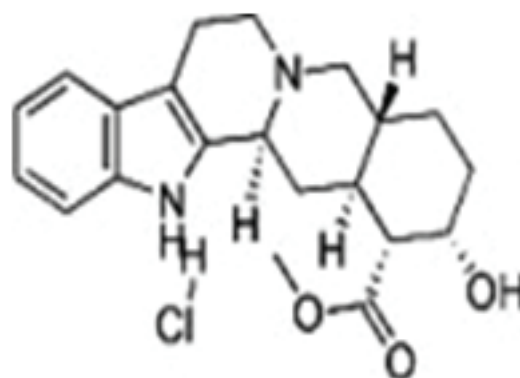


Fig. 1. Chemical structure of Yohimbine.

athletes and as an aphrodisiac. Extracts from yohimbe are applied as dietary supplements for improving sexual function [1].

Yohimbine has high affinity for α₂ adrenergic receptors, moderate affinity for the α₁ receptors, 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1F}, 5-HT_{2B}, D₂ receptors and weak affinity for the

5-HT_{1E}, 5-HT_{2A}, 5-HT_{5A}, 5-HT₇, D₃ receptors. It behaves as an antagonist of α_1 adrenergic, α_2 adrenergic, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2B}, D₂ receptors and as a partial agonist of 5-HT_{1A} receptors [2].

Depending on dosage, Yohimbine can either increase or decrease systemic blood pressure respectively through vasoconstriction or vasodilation. Due to the highest affinity for the α_2 receptors, in small doses increases blood pressure by causing a relatively selective α_2 blockade. At higher doses an α_1 blockade can occur leading to a potentially dangerous reduction of blood pressure [2].

Yohimbine is selective for the presynaptic α_2 receptors [3], enhances plasma testosterone levels and decreases body fat [4].

Yohimbine is applied for treatment of sexual dysfunctions [5] as erectile [6] and psychogenic impotence [7] and in nonorganic [8] and organic [9] erectile dysfunction.

Yohimbine has been used as fat loss strategy by many athletes [10]. Yohimbine decreases the activity of the sympathetic nervous system and increases the activity of the parasympathetic nervous system [3]. Because the use of herbal food supplements is increasing, the analytical control of these products is essential for customer safety.

Different methods are described for analysis of Yohimbine: HPLC, gas chromatography, spectrophotometric, voltammetric and polarographic methods. For qualitative and quantitative analysis of Yohimbine in commercial supplements the most often reported methods are: 1) HPLC-UV-MS with atmospheric pressure chemical ionisation and electrospray ionisation [11]; 2) HPLC-quadrupole time-of-flight (Q-TOF MS) and with triple quadrupole (QQQ MS) [12]; 3) ultra performance liquid chromatography with UV- and mass detection on C₁₈ column [13] and gradient elution with mobile phases: 0.1 % (v/v) aqueous ammonium hydroxide and 0.1 % ammonium hydroxide in methanol [14].

Yohimbine hydrochloride is analysed by isocratic HPLC by using a column C₁₈ (150 mm x 4.6 mm x 5 μ m), flow rate: 1.0 ml/min. and UV-

detection at $\lambda = 270$ nm and mobile phase: methanol : water = 70 : 30 v/v [15] or methanol : water = 45 : 55 v/v [16].

For separation and simultaneous determination of Yohimbine, Sildenafil, Vardenafil and Tadalafil in dietary supplements is used HPLC-MS/MS on a C₁₈ column with gradient elution, mobile phase consisting of acetonitrile and 0.1 % acetic acid aqueous solution and flow rate: 0.5 ml/min. [17]. HPLC with amperometric detection is described for quantitation of Yohimbine in biological fluids [18]. Other applied methods are gas chromatography with mass spectrometry [19] and amperometry using a boron-doped diamond electrode [20].

The aim of current work was the validation of HPLC method with UV-detection for identification and determination of Yohimbine in Yohimbine containing products (supplements and drugs).

Materials

I) Reference standard: Yohimbine with purity > 98 %.

II) Reagents with analytical grade quality: acetonitrile for HPLC (Sigma Aldrich, N: SZBD 150 SV UN 1648), methanol for HPLC ≥ 99.9 % (Sigma Aldrich, N: SZBD 063 AV UN 1230), distilled water.

Methods: HPLC method

I. Chromatographic system: Liquid chromatograph Shimadzu (Japan) (LC-10 Advp), equipped with: column Spherisorb ODS RP C₁₈, column oven (CTO-10 Asvp); isocratic pump (LC-10 A); 20 μ l injector loop; UV-VIS detector at fixed wavelengths (SPD-10 Avvp).

II. Chromatographic conditions: stationary phase: column Spherisorb ODS RP C₁₈ (250 mm x 4.6 mm x 5 μ m), column temperature: 25 °C, mobile phase: methanol : acetonitrile : water = 70 : 20 : 10 v/v, flow rate: 2 ml/min., UV-detection at $\lambda = 270$ nm. Before using mobile phase was filtered through membrane filter with pore size 0.45 μ m.

III. Preparation of standard solutions of Yohimbine.

For validation of method for analytical parameter linearity an accurately weighed quantity of reference standard Yohimbine: 5 mg, 10 mg, 20 mg, 30 mg, 40 mg, 50 mg, 70 mg, 100 mg was dissolved in separate volumetric flask of 100.0 ml in acetonitrile. Aliquot part of 1 ml of every solution was diluted to 100.0 ml volumetric flask to obtain solutions with concentration correspondingly: $5 \cdot 10^{-7}$ g/ml; $1 \cdot 10^{-6}$ g/ml; $2 \cdot 10^{-6}$ g/ml, $3 \cdot 10^{-6}$ g/ml, $5 \cdot 10^{-6}$ g/ml, $7 \cdot 10^{-6}$ g/ml, $1 \cdot 10^{-5}$ g/ml.

IV. Statistical method of root mean square error (RMSE)

For determination of limit of detection (LOD) and limit of quantification (LOQ), the data for peak's area under the curve obtained from chromatograms of standard solutions with increasing concentration are subjected to linear regression analysis. The regression equations $y = ax + b$ and the coefficient of linear regression are obtained. From the regression equation $y = a \cdot x + b$ is calculate the predicted area under the curve (A_p). For each sample are calculated:

$$E = |A_p - A|, E^2 = [|A_p - A|]^2, E1 = \frac{\sum E^2}{n - 2};$$

$$RMSE = \sqrt{E1};$$

$$LOD = 3 \cdot RMSE/a;$$

$$LOQ = 10 \cdot RMSE/a [21].$$

Results and discussion.

HPLC method was validated for analytical parameters: selectivity, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy and precision as per ICH guidelines [21].

I. Selectivity.

In the same manner like the standard solution, placebo solution, without the active substance Yohimbine was prepared. The selectivity of the applied HPLC method was confirmed by the fact, that on chromatograms with "placebo" preparation did not exist peaks with t_R , corresponding to t_R of Yohimbine in standard solutions: 2 ml/min.: $t_R = 3.046$, SD = 0.11; 1 ml/min.: $t_R = 6.149$ (SD = 0.07, RSD = 1.14 %).

II. Linearity.

For the estimation of the relationship between the concentration and the area of the chromatographic peak, were obtained chromatograms of a series of standard solutions of Yohimbine in concentration range: $5 \cdot 10^{-7}$ g/ml ÷ $1 \cdot 10^{-5}$ g/ml.

Linear regression analysis was performed. The regression calibration curve was built through the application of linear regression analysis. Linearity accordance between the concentration and peak area in range: $5 \cdot 10^{-7}$ g/ml ÷ $1 \cdot 10^{-5}$ g/ml was proved by the regression equation: $y = 94517560831 \cdot x + 25119$. The calculated correlation coefficient R^2 is 0.9981. Parameter of regression equations are presented on Table 1.

Table 1. Parameter of regression equation for Yohimbine.

N:	Parameter	Results
1.	Range [g/ml]	$5 \cdot 10^{-7}$ g/ml ÷ $1 \cdot 10^{-5}$ g/ml
2.	Regression equation	$y = 94517560831 \cdot x + 23119$
3.	Slope	94517560831
4.	Intersept	25119
5.	Correlation coefficient (R^2)	0.9981

On Fig. 2. is illustrated the calibration curve, which presents the linear dependence between the concentration and peak area in range: $5 \cdot 10^{-7}$ g/ml ÷ $1 \cdot 10^{-5}$ g/ml.

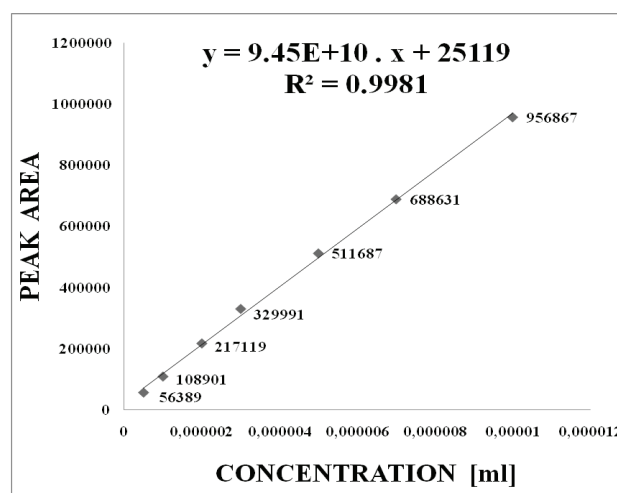


Fig. 2. Linearity for Yohimbine.

Table 2. Concentration (C), peak area (A), peak high (H) for standard solutions of Yohimbine.

N:	t_R [min.]	C [g/ml]	A	H	Ap	$ A_p - A $	$[[A_p - A]]^2$
1.	2.944	1.10^{-5}	956867	26226	970295	13428	180311184
2.	2.947	7.10^{-6}	688631	19829	686742	1889	3568321
3.	2.987	5.10^{-6}	511687	14365	497707	13980	195440400
4.	3.152	3.10^{-6}	329991	8777	308672	21319	454499761
5.	3.148	2.10^{-6}	217119	5607	214154	2965	8791225
6.	3.176	1.10^{-6}	108901	2992	119637	10736	115261696
7.	2.965	5.10^{-7}	56389	2435	72378	15989	255648121
$\sum E^2 = 1213520708$							
$E1 = \frac{\sum E^2}{n-2} = \frac{1213520708}{5} = 242704142$							
$RMSE = \sqrt{242704142} = 15579$							

On Table 2. for standard solutions of Yohimbine are presented data for: t_R [min.], concentration (C), peak area (A), peak high (H), calculated from regression equation peak area (Ap),

error: $E = |A_p - A|$, $E^2 = [[A_p - A]]^2$, $E1 = \frac{\sum E^2}{n-2}$;
 $RMSE = \sqrt{E1}$, $LOD = 3.RMSE/a$;
 $LOQ = 10.RMSE/a$ [21].

III. Limit of detection (LOD) and limit of quantification (LOQ).

The limit of detection (LOD) and limit of quantification (LOQ) were obtained by calculating using the standard formula as per the ICH guidelines.

LOD is the smallest concentration of the analyte that gives the measurable response and was calculated as a ratio between SD of the response (15579) and the slope of calibration curve (94517560831).

$$LOD = \frac{3.3 \times 15579}{94517560831} = 5.44 \cdot 10^{-7} \text{ g/ml}$$

LOQ is the smallest concentration of the ana-

lyte, which gives response that can be accurately quantified.

$$LOQ = \frac{10 \times 15579}{94517560831} = 1.65 \cdot 10^{-6} \text{ g/ml}$$

IV. Accuracy and precision (repeatability).

For the estimation of analytical parameters accuracy and precision (repeatability) 6 equal standard solutions of Yohimbine with concentration 1.10^{-6} g/ml are analysed by the written HPLC method.

On Table 3. are summarized results for: peak area (A); UA – Chauvenet's criterion for area; C – obtained quantity of Yohimbine by method of calibration curve; UC – Chauvenet's criterion for obtained content; RC [%] – degree of recovery; t_R – retention time.

On Table 3. are indicated: N – number of the individual measurements (1 ÷ 6); \bar{X} – arithmetical mean; SD – standard deviation; RSD – relative standard deviation (%); $S \bar{X}$ – mean quadratic error; P – confidence possibility (%); t – coefficient of Student; $\bar{X} \pm t.S \bar{X}$ – confidence interval; E [%]

Table 3. Peak area (A), quantity (C), Chauvenet's criterion for A and C of Yohimbine, degree of recovery (R) and retention time (t_R).

N:	A	UA	C	UC	RC [%]	t _R [min.]
1.	115948	1.30	9.61	1.30	96.1	3.173
2.	116838	0.83	9.70	0.85	97.0	3.049
3.	117901	0.26	9.82	0.25	98.2	3.176
4.	118801	0.21	9.91	0.2	99.1	2.945
5.	119951	0.82	10.03	0.8	100.3	3.113
6.	120965	1.36	10.14	1.35	101.4	3.187
\bar{X}	118401		9.87		98.68	3.107
SD	1889		0.2		2.0	0.1
RSD [%]	1.6		2.03		2.03	3.22
s \bar{X}			0.08		0.82	
P [%]			98.0		98.0	
t			3.37		3.37	
t.S \bar{X}			0.27		2.76	
$\frac{\bar{X} - t.S \bar{X}}{\bar{X} + t.S \bar{X}}$			9.6 ÷ 10.14		95.92 ÷ 101.44	
E [%]			0.81		0.83	

– relative error [22].

Analytical parameter accuracy is presented by the degree recovery R (%) ± RSD (%) [22]. All results suit respective confidence interval: 98.68 % ± 2.03 %.

For the assessment of precision is calculated sample standard deviation (SD) and relative standard deviation (SD), by the applying of the Bessel's correction, in which the denominator N – 1 (degrees of freedom) is used instead of N and in this case (S)² is an unbiased estimator for (SD)².

Precision is estimated by the uncertainty of the result, determined by standard deviation (SD), relative standard deviation (RSD) and confidence interval ($\bar{X} \div t.S \bar{X}$). Results from Table 3. showed that at confidence possibility P = 98 % (t = 3.37), all data for content of Yohimbine are included in respective confidence interval:

9.6 mg ÷ 10.14 mg.

For all of the obtained results for spot area is necessary to estimate the Chauvenet's criterion (U), because when U for one value is higher than the relevant standard criterion (USt), the result must be removed as unexpected. The relation $U < 1.73$ (Table 2.) show, that all experimental results for UA are lower, than standard requirement: $U_{max} = 1.73$ (N = 6) and it isn't necessary to remove data for peak area.

IV. Test for system suitability.

The suitability of the system is confirmed by the lack of a statistically significant difference between the values of the chromatographic parameter retention time (t_R) (Table 4.) in the analysis of standards of Yohimbine: 2 ml/min: t_R = 3.046 min. (SD = 0.11); 1 ml/min.: t_R = 6.149 (SD = 0.07, RSD = 1.14 %).

Table 4. Retention times for Yohimbine – estimation of system suitability.

N:	C [g/ml]	t _R [min.] 2 ml/min.	t _R [min.] 1 ml/min.
1.	1.10 ⁻⁵	2.944	6.165
2.	7.10 ⁻⁶	2.947	6.085
3.	5.10 ⁻⁶	2.987	6.047
4.	3.10 ⁻⁶	3.152	6.241
5.	2.10 ⁻⁶	3.148	6.145
6.	1.10 ⁻⁶	3.176	6.120
7.	5.10 ⁻⁷	2.965	6.241
$\bar{X} \pm SD$		3.046	6.149
SD		0.11	0.07
RSD [%]		3.61	1.14

Conclusion.

The proposed validated HPLC method is appropriate for quality control of Yohimbine in Yohimbine containing products.

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