Journal of Advanced Pharmaceutical Sciences

Journal homepage: https://japs.journals.ekb.eg/

Research article

Development of a validated HPTLC-dual wavelengths method for simultaneous Determination of yohimbine, caffeine, niacin and alpha-tocopheryl succinate in their challenging quaternary mixture

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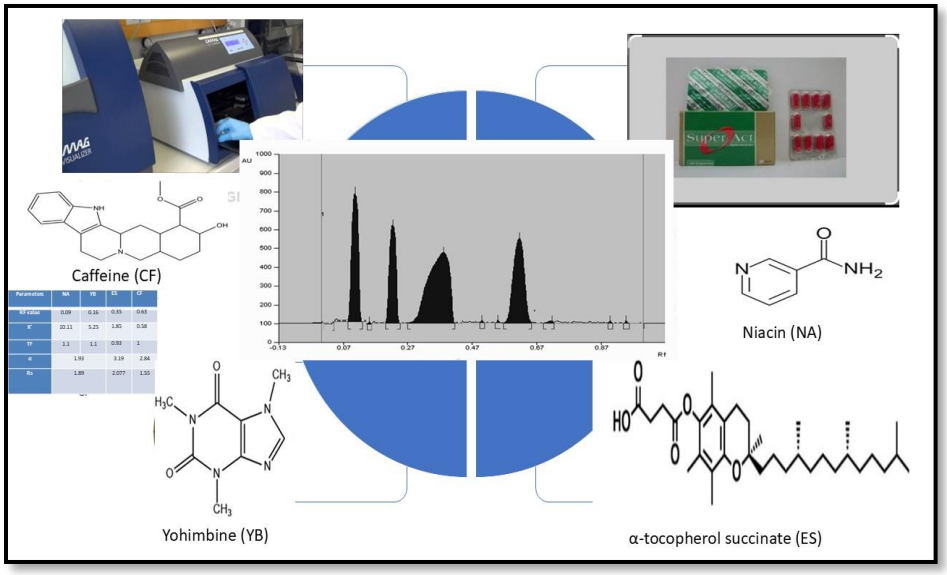
Abstract:



Millions of men throughout the world are endangered by erectile dysfunction (ED). Diabetes, vascular disease, neurological disorders, or prostate-related medications can all lead to ED. Sometimes it is as simple as the side effect of a specific treatment. However, the reason is more complicated for 75% of men. The treatment for ED could include certain prescription medications, lifestyle changes and other natural treatments. Yohimbine (YB) is an indole alkaloid derived from Central African yohimbe tree bark that is widely used as therapy for ED. In the Egyptian market, YB has been formulated as capsules in combination with Caffeine (CF), Niacin (NA) and Alpha-tocopherol succinate (ES). This quaternary mixture is effectively used for treatment of ED and other associated disorders like fatigue, heart attack, leg pain due to blocked arteries and vitamin B3 deficiency. So, it seems necessary to develop selective analytical technique for assay of this multi-combination. In the proposed study, validated HPTLC technique was developed for concurrent determination of YB, CF, NA and ES, formulated in Super act® capsules.

Received: 22-11-2024 Accepted: 30-12-2024 Published: 6-1-2025

ISSN: 3009-7061 **DOI: 10.21608/japs.2024.334707.1031**

The suggested method depends on HPTLC segregation of the drugs at 270 nm for YB, CF, NA and at 280 nm for ES. Separation was accomplished on silica gel 60 F254 aluminum sheets, produced by Merck, using chloroform: methanol (9.8:0.2 v/v) as mobile phase. The method was found linear in the range of 0.8-6, 0.8-10, 0.4-6 and 6-14 ug /band^{-1} for YB, CF, NA and ES, respectively. The reported chromatographic method performed well in terms of linearity, sensitivity, precision, accuracy, and stability after undergoing validation in accordance with ICH guidelines. Using the F and t-test as statistical methods, it was effectively utilized to analyze quaternary mixtures in dosage form, and the outcomes showed good compliance with those from the comparison method.

Keywords: HPTLC, Quaternary mixture, Dual wavelength, Combination therapy.

1. Introduction

The treatment for Erectile dysfunction (ED) may include certain prescription medications, lifestyle changes and other natural treatments. Sometimes ED is as simple as the unfavorable influence of a certain medication. Nevertheless, the main cause is more intricate for 75% of men.

Yohimbine hydrochloride (YB) is an indole alkaloid of the African yohimbe tree bark. The Central release of norepinephrine or epinephrine is induced by YB which is specific for the presynaptic alpha-2 receptor. This central action raises sexual arousal. YB also partially antagonizes contraction of corporeal cavernosal smooth muscle induced by norepinephrine [(1)].

Caffeine (CF) is a non-selective inhibitor of phosphodiesterase. It induces relaxation of the penile helicine arteries, and the cavernous smooth muscle, thus increasing penile blood flow [(2,3)]. Niacin (NA) is a lipid-lowering substance that has the ability to raise HDL cholesterol levels in the blood, which improves the lipid profile. Niacin may help with atherosclerosis and endothelial function, according to studies. Niacin can enhance the erectile function in patients of dyslipidemia. [(4)]. Vitamin E (α-tocopherol succinate) (ES) is a lipophilic antioxidant which improves endothelial cell function, traps oxygen free radicals, and inhibits monocyte endothelial adhesion and cytokine release. Moreover, some reports reveal the platelet aggregation hinderance by a protein kinase C-dependent mechanism. Additionally, vitamin E supports protein stability and cell membrane integrity while promoting NO-mediated arterial relaxation. ES was shown to increase intra-cavernosal pressure, protect penile tissue, and maintain the function of nerve in ageing [(5)].

Mixture of YB, CF, NA, and ES medication is formulated as Super act® capsules which is used in the treatment of male sexual dysfunction associated with diabetic and vascular disorders. So, it deems necessary to develop a selective analytical technique for the assay of this multi-combination. Only one spectrophotometric method was reported to separate the quaternary mixture [(6)]. Due to its benefits of high sample throughput, low expenses for operation, and minimal sample cleaning requirements, HPTLC has developed into a routine valid analysis method [(7-11)]. The main advantage of HPTLC over other chromatographic methods is that, in contrast to HPLC, multiple samples can run concurrently with a minimal amount of mobile phase, which lowers analytical time and cost.

Literature review data lacks any chromatographic method for assay of reported mixture. The challenge of this work was to perform a validated and simple HPTLC method for separation and quantification of YB, CF, NA and ES in

ISSN: 3009-7061 **DOI: 10.21608/japs.2024.334707.1031**

laboratory prepared mixture and pharmaceutical dosage form.

The developed technique was validated for linearity, limits of detection, quantification, precision, accuracy, and specificity in accordance with ICH guidelines. The Chemical structures of the investigated drugs were displayed in **Fig. 1**.

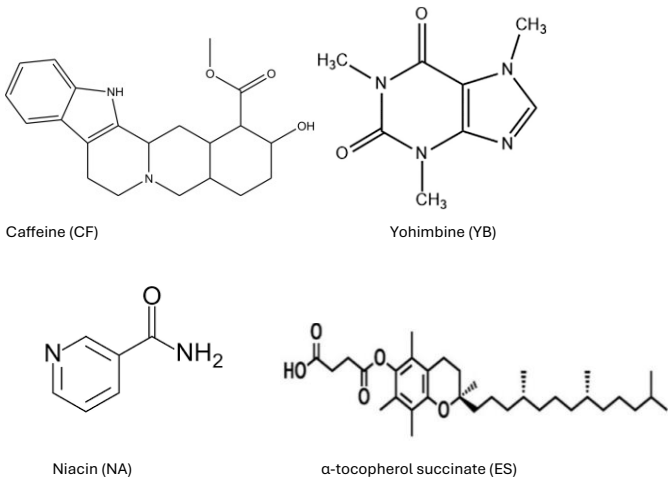


Fig. 1: The chemical structures of the studied drugs

2. Experimental

2.1 Materials and reagents

Amirya Pharmaceuticals (Pharco Company) (Egypt) presented Caffeine Anhydrous powder as a gift. α-tocopheryl succinate and nicotinamide were supplied by VitaBiotics Egypt for pharmaceutical industries. Yohimbine hydrochloride alkaloid was gifted from the Pharmacognosy department, Alexandria University. Analytical grade ethanol was purchased from International Company for supplements and medicine industries. Analytical grade Methanol was purchased from Fisher scientific. Ltd., India. Analytical-grade chloroform was obtained from SD fine-chem limited, India.

2.2 Instrumentation

Linomat IV automatic applicator (Camag, Switzerland) with 100 ul micro-syringes. 20*20 cm Camag chamber, WinCats software operated Camag HPTLC scanner and pre-coated silica gel HPTLC plates 60 F254 (15*10 cm) layer, Merck (Germany, Darmstadt) were utilized.

2.3 Standard solutions preparation

In ethanol, different stock solutions of YB, CF, NA, and ES at a concentration of 2 mg/ml were prepared. To obtain concentration ranges of each drug as in **Table 1**, working solutions were made by accurately measuring volumes of stock solutions of each drug into four 10-mL sets of volumetric flasks and completing with ethanol. To construct calibration graphs for each drug, the peak area of each solution was plotted versus the concentration of solution.

2.4 Chromatographic conditions

Standard solution for each drug was made, and bands of the standard solutions were applied to an HPTLC plate with a fixed volume of 20 ul, a band width of 6 mm, and a distance of 10 mm from the bottom of the plate. To separate the investigated drugs in ascending order of increasing polarity of the studied drugs in mobile phase, the mobile phase vapour (chloroform: methanol in ratio 9.8:0.2 by volume) was saturated into the twin trough glass chamber for 30 min. After being air-dried, the plate was detected at 270 nm for YB, CF, and NA and 280 nm for ES chosen according to the absorption spectra of the drugs shown in **Fig. 2** [(6)]. The calibration graphs were prepared by plotting the peak area for each drug (at its specific wavelength) against its matching concentration.

2.5 Analysis of synthetic mixtures

In separate sets of 10-mL volumetric flasks, accurately transfer portions of YB, CF, NA, and ES standard solutions were added. Afterwards, ethanol was added to the volume to produce two different synthetic combinations; **Table 2**. Peak area of each drug in the prepared mixtures was measured.

ISSN: 3009-7061 **DOI: 10.21608/japs.2024.334707.1031**

Table 1: Linearity parameters of the proposed HPTLC method for simultaneous determination of YB, CF, NA& ES.

Parameters	YB	CF	NA	ES
Linearity range	0.8-6	0.8-10	0.4-6	6-14
LOD	0.102	0.524	0.248	2.257
LOQ	0.308	1.589	0.752	6.839
Intercept	2598.396	16887.272	2763.147	-4836.24
Slope	691.093	1934.441	1644.061	2255.79
Correlation coefficient (r)	0.9998	0.9989	0.9992	0.9936
Sa^b	21.287	307.308	123.5599	1542.781
Sb^b	6.9878	50.570	38.373	148.454
Sy/x^b	29.315	397.007	167.582	938.907
F	9781.258	1463.256	1835.5901	230.894
Significance F	$2.279x10^{-6}$	$3.9303x10^{-5}$	$2.7987x10^{-5}$	$6.2\ x10^{-4}$

a Concentration in µg /band

b Sa is standard deviation of intercept, Sb is standard deviation of slope, and Sy/x is standard deviation of Residuals.

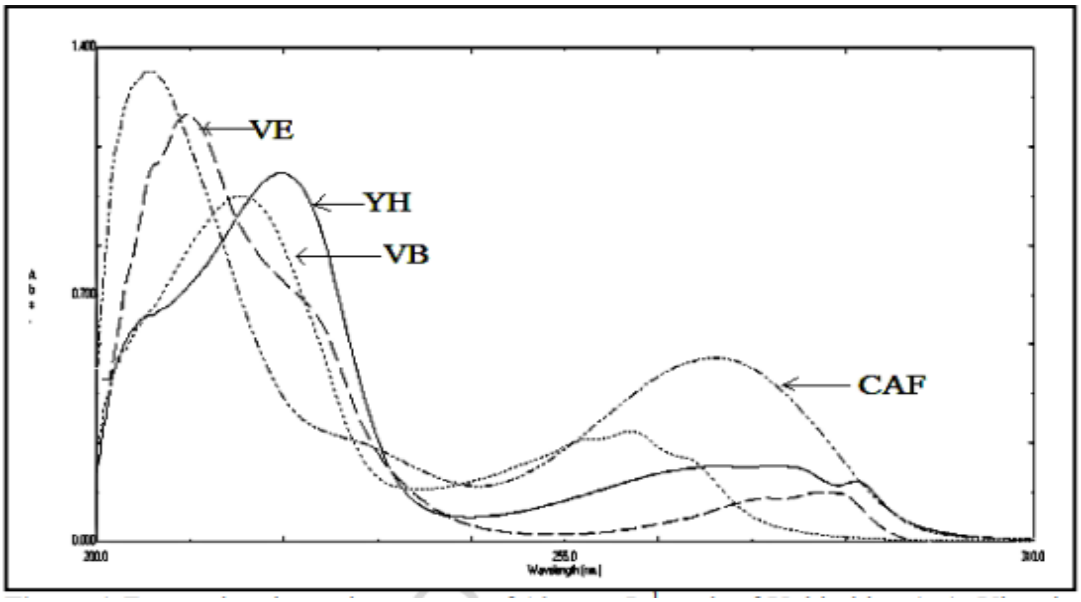


Fig. 2: Zero -order absorption spectra of NA, YB, ES and CF using methanol as blank

Table 2: Simultaneous analysis of CF, YB, NA& ES in synthetic mixtures using the proposed HPTLC method (n = 3).

	Mean% recovery± SD			
YB: CF: NA: ES (µg /band)	YB	CF	NA	ES
1:5:1:3	99.77±0.399	100.306±0.23	101.853±0.492	100.277±0.602
1:1:1:1	100.06 ± 0.806	100.07±0.06	100.73±0.56	98.857±1.28

ISSN: 3009-7061 **DOI: 10.21608/japs.2024.334707.1031**

2.6 Analysis of pharmaceutical dosage form

The capsules were labelled to contain 5.4 mg, 25 mg, 5 mg, and 15 mg for YB, CF, NA, and ES, respectively. In order to prepare laboratory-made capsules, 2-mg/ml solution of each drug was prepared in a 10-mL volumetric flask in ethanol. A 0.45-µm membrane filter was used for filtering the solutions. Aliquots from each solution were mixed and diluted with ethanol to achieve the ratio of the four drugs mentioned on the capsules. The mixture working solution was chromatographed and recoveries were computed.

3. Results and discussion

The objective of presented work is to propose a selective HPTLC approach for the separation of the investigated quaternary combination using a simplified development system. The suggested technique is a simple and selective approach for achieving this separation of the drugs at wavelengths. CF is more soluble in chloroform than ES, therefore CF showed higher RF than ES [(12)]

3.1 Method development and optimization

Checking the impact of numerous variables was important in order to optimize the approach conditions [(13)]. To separate the quaternary mixture from each other's, Different developing systems were investigated. Many trials were performed using different ratios from mobile phase such as Chloroform 100% where YB and NA didn't move up and remained at the baseline. Also, Chloroform: isopropanol with ratio (9.8:0.2) was tried but ES was almost at the solvent front and it was split. Not only these trials but also, chloroform: methanol with ratio (9.5:0.5) was used and this showed YB moving extensively up the plate and near the solvent front with no obvious difference between RF of YB and CF. However, despite employing ammonia to modify pH, no suitable separation could be obtained. Chloroform: methanol: ammonia with ratio 9.8:0.2:0.1 showed tailing of ES and 9.8:0.2:0.5 showed ES at the solvent front and when applying trials using 0.2, 0.3 and 0.4 ammonia didn't enhance the separation or peak shape compared to its absence. Certainly, it was settled that the greatest separation of the cited drugs was accomplished by applying the developing system using chloroform: methanol (9.8:0.2, by volume). Rf for YB, CF, NA and ES were 0.16+ 0.01, 0.63+0.01, 0.09+0.01, and 0.35+0.02, respectively.

At room temperature, well-defined spots were obtained when mobile phase saturated the chamber for at least 45 min. To prevent the edge effect, which could result in irreproducible RF values, and to remove uneven solvent evaporation losses from the plate, the chamber had to be saturated.

Several scanning wavelengths were tested; on using Dual wavelength 270 and 280 nm, the separated peaks were sharp, more sensitive with lowest degree of noise, as shown in **Fig. 3**. However, according to the obtained chromatograms, the drugs peaks were tailed, overlapped and less obvious using other tested wavelengths (216, 257, 273, 291 nm).

3.2 System suitability parameters

To estimate the performance of the chromatographic method, system suitability parameters were calculated. The resolution (Rs), tailing factor (T), capacity factor (K'), and selectivity factor (α) were calculated to verify that the system has applicability, and acceptable results were attained as shown in **Table 3** [(14)]. It is important to highlight that NA has a comparatively low RF value (0.09). The polarity of drug in comparison to other drugs may be the reason for this; as a result, it was kept on the silica plate and displayed an early band. Since, CF is more soluble in chloroform than ES, it displayed a larger RF value. the resolution between YB and NA was fair due to their similar chemical structures.

ISSN: 3009-7061 **DOI: 10.21608/japs.2024.334707.1031**

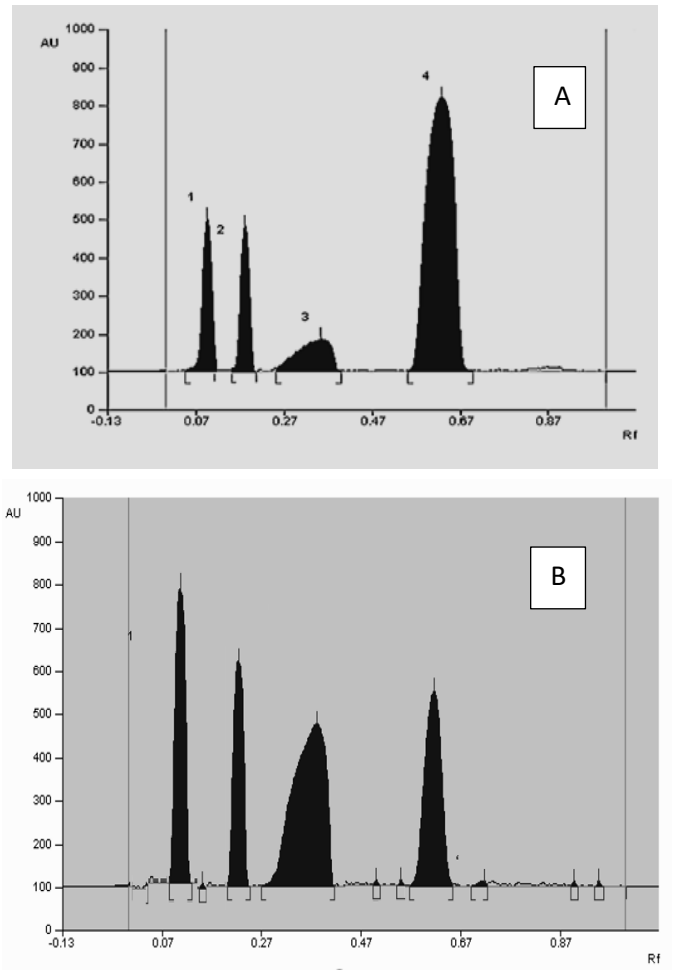


Fig. 3: HPTLC densitograms of the separated peaks of (1) NA, (2) YB, (3) ES and (4) CF with detection at (**A)** 270 nm (**B)** 280 nm using chloroform: methanol (9.8:0.2 v/v) as the developing system

3.3 Method validation

The analytical procedure was validated as per the ICH guidelines [13], in order to assess the performance of the developed method.

3.3.1 Linearity and range

The linearity of the suggested method was validated by constructing various calibration curves.

The proposed method's linearity was validated by creating various calibration curves. A range of standard drug solutions were analyzed, and the calibration graphs between peak areas and corresponding band concentrations were displayed **Fig. 4**. Regression analysis was applied, and analytical parameters were computed. The linear concentration ranges and other statistical variables for the proposed method were listed in **Table 1** [15]. The closer the points are to the straight line, the lower the standard error of the estimate. Using the suggested measurement techniques, the standard deviations of the intercept (Sa) and slope (Sb) are also provided for each compound. Good linearity of the calibration graphs is indicated by the correlation coefficients and F-values [16, 17]. When the variance ratio (F-values) rises for equal degrees of freedom, the mean of squares resulting from regression increases and the mean of squares resulting from residuals decreases. The steepness of the regression line will increase with the increase in mean of squares due to regression. Accordingly, regression lines with low significance F (high F-values) are significantly inferior to those with low F-values. High (r) and (F) values are displayed by good regression lines

Table 3: System suitability parameters of the proposed HPTLC method of YB, CF, NA and ES.

Parameters	NA	YB	ES	CF	Reference value
RF value (retardation value)	0.09	0.16	0.35	0.63	
K'(capacity factor)	10.11	5.25	1.85	0.58	0-10
TF (tailing factor) [a]	1.1	1.1	0.93	1	~ 1
α (selectivity factor) [b]	1.93	3.19	2.84		α>1
Rs (chromatographic resolution) [c]	1.89	2.077	1.55		Rs>1.5

a TF = W0.05/2f, where W0.05 is the width of the peak at 5% height and f is the distance from peak maximum to the leading edge of peak

b α = k´2 / k´1, where k´ is the capacity factor; k´ = (1 – RF) / RF

c Rs = (2 (tr2 – tr1)) / (W1 + W2), Where tr is retention time and W is peak width at baseline of the peak

ISSN: 3009-7061 **DOI: 10.21608/japs.2024.334707.1031**

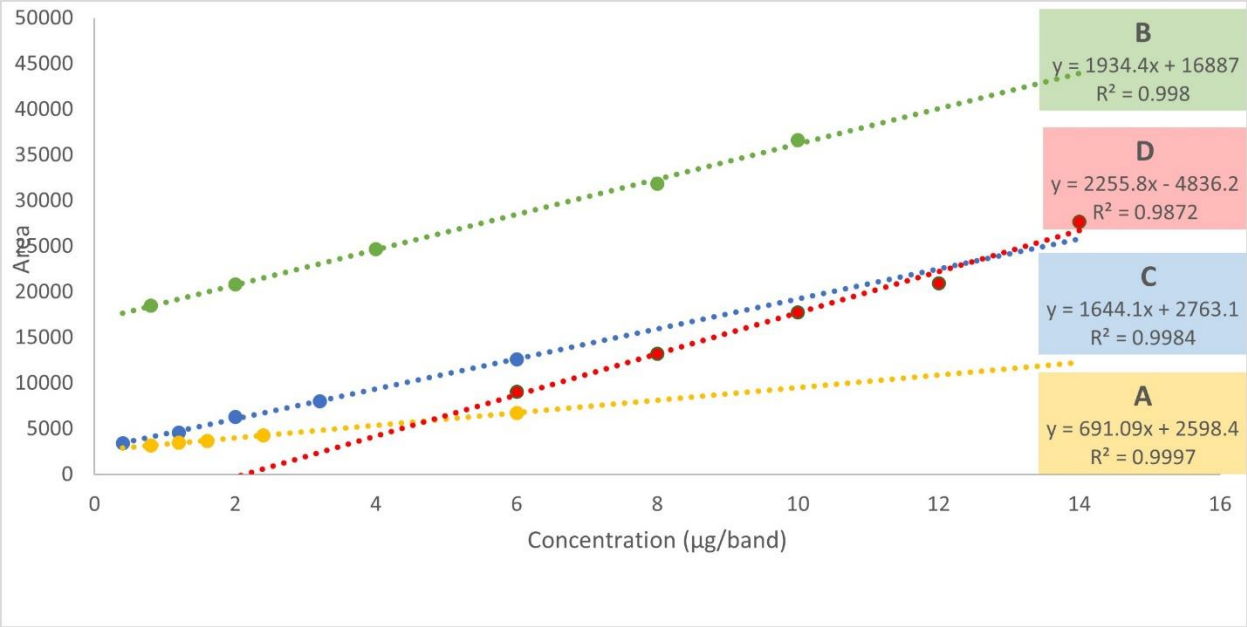


Fig. 4: Calibration curves of **A** yohimbine 0.8-6 µg/band (yellow line), **B** caffeine 0.8-10 µg/band (green line), **C** Niacin 0.4-6 µg/band (blue line), **D** Alpha tocopherol succinate 6-14 µg/band (red line)

3.3.2 Limit of detection (LOD) and limit of quantification (LOQ)

Drug concentrations, showing signal — to — noise ratio 2 1(LOD) and 10:1(LOQ), were measured. The determined data for each drug were cited in **Table 1**

3.3.3 Accuracy

Triplicate measurements of various strengths of synthetic mixture were made. The results in **Table 4** show satisfied values of % recovery and % error. This ensures the satisfactory accuracy of suggested HPTLC method.

3.3.4 Precision

Precision was assessed by triplicate measurement of differing strengths of YB, CF, NA and ES in synthetic combination. The obtained results show %RSD < 2% for both intra-day and inter-day precision **Table 4**. This indicates that the suggested methodology is highly precise and repeatable.

3.3.5 Selectivity

Selectivity was confirmed by testing different mixtures of cited drugs in different ratios within the proposed linearity range. Accepted selectivity results were shown in **Table 2**. [15]. The same solvent system was employed in two-dimensional chromatography to detect any decomposition that might have taken place during spotting and development. Peak(s) of the decomposition product(s) must be obtained for the analyte in both the first and second directions of the run if decomposition takes place during development. The stability of each drug in solutions was demonstrated by the absence of decomposition during spotting and development under the suggested conditions. Following the standard process of developing the plate using chloroform: methanol (first development), it was dried, turned 90 degrees, and then developed again using the same mobile phase. On the diagonal line, the spot was located. This shows that the spot is stable throughout the chromatographic process.

3.3.6 Stability in solutions

Using the steps of the suggested HPTLC approach, solutions of YB, CF, NA, and ES

ISSN: 3009-7061 **DOI: 10.21608/japs.2024.334707.1031**

were prepared, kept at ambient temperature for 0.5, 1.0, 1.5, 2, and 2.5 hours, and then analyzed. The chromatograms of the results show absence of additional peaks. It indicates that YB, CF, NA, and ES are all rather stable

3.4 Application to pharmaceutical dosage form

The four drugs in their lab-made capsules were successfully examined using the suggested HPTLC method. The outcomes indicated that the value for % recovery and %RSD were satisfactory.; **Table 5**. The student's t-test and the variance ratio F-test were used to compare results with those from the previously published spectrophotometric method [6]; P=0.05 [15]. The t and F values that had been obtained were below threshold levels.; **Table 5**; a high extent of agreement between the suggested and reported approaches.

3.5 Comparison to reported method

The proposed method outperforms the reported one in several aspects. The present method is simpler and offers less complicated mathematical operations than spectrophotometric one which is time consuming and requires large quantity of sample and solvents. Also, the proposed methods provide optimum selectivity without any necessity for convoluted operations.

4. Conclusion

Successful separation of YB, CF, NA, and ES, in their dosage form and laboratory prepared mixtures, using dual wavelength HPTLC method was accomplished. The suggested method is selective, valid, cost-effective and does not need complicated instrumentation and is time saving. These benefits advocate the use of proposed methods in regular and quality control analyses.

Table 4: Evaluation of the proposed method's accuracy and precision for simultaneous determination of CF, YB, NA& ES using standard addition method (n = 3)

Concentration (µg /band)				Recovery % ±DS[a]				RSD%[b]				Er%[c]			
YB	CF	NA	ES	YB	CF	NA	ES	YB	CF	NA	ES	YB	CF	NA	ES
Accuracy and intra-day precision															
0.8	0.8	0.4	6	98.30±1.02	101.90±1.53	100.47±0.85	101.92±0.38	1.03	1.50	0.85	0.37	-1.70	1.90	0.47	1.92
2.4	4	3.2	10	100.85±0.99	101.01±0.69	99.77±0.22	100.01±0.08	0.98	0.68	0.22	0.08	0.85	1.01	-0.23	0.01
6	10	6	14	100.26±0.92	101.29±1.26	99.82±0.24	101.92±0.96	0.92	1.24	0.24	0.95	0.26	1.29	-0.18	1.92
Accuracy and inter-day precision															
0.8	0.8	0.4	6	99.58±1.39	101.27±0.96	100.31±0.75	101.37±0.52	1.40	0.95	0.75	0.51	-0.42	1.27	0.31	1.37
2.4	4	3.2	10	100.85±0.86	100.95±0.55	99.90±0.20	99.99±0.10	0.85	0.54	0.20	0.10	0.85	0.95	-0.10	-0.01
6	10	6	14	99.95±0.64	101.62±0.68	99.81±0.22	101.42±0.65	0.64	0.67	0.22	0.64	-0.05	1.62	-0.19	1.42

a is standard deviation, b is percentage relative standard deviation and c is percentage relative error

Table 5: Assay of YB, CF, NA& ES in their Laboratory prepared capsule using the proposed HPTLC method (n = 6).

Laboratory prepared capsule[a]	Mean% recovery ± SD		t[b]	F[b]
	Proposed method	Reported method [6]		
YB	99.915±0.591	100.53±0.480	1.8778	1.5140
CF	100.188±0.199	100.24±0.329	0.3346	2.7425
NA	101.29±0.776	100.4± 0.815	1.9368	1.1020
ES	99.567±1.185	100.56±0.611	1.8251	0.2659

a Labelled to contain 5.4, 25, 5 and 15 mg per capsule YB, CF, NA and ES, respectively.
b Theoretical value of t and F at p = 0.05 are 2.23 and 5.05, respectively.

ISSN: 3009-7061 **DOI: 10.21608/japs.2024.334707.1031**

Conflict of interests

No competing interests as declared by the authors.

Availability of data and materials

Data and materials are illustrated in detail within the manuscript. For further information, please contact the authors.

Funding

The authors declared that this work was not funded by any agencies.

Authors' contributions

Ass. Prof. Dina A. Gawad: Conceptualization, Methodology, Writing review and editing, Supervision.

Prof. Rasha M. Youssef: Conceptualization – Writing review and editing, Formal analysis, Supervision.

Aya M. Abdelhafez: Methodology, Data analysis, Writing original draft.

Prof. Ekram M. Hassan: Conceptualization, Data curation, Supervision.

Acknowledgements

The authors acknowledge the Science Park Unit at the Faculty of Pharmacy, Alexandria University

Highlights

- **Yohimbine** was formulated in combination with Caffeine, Niacin and Alpha-tocopherol succinate for treatment of Erectile dysfunction.
- The proposed dual-wavelength HPTLC method was developed for simultaneous determination of the four drugs.
- The proposed chromatographic approach showed good performance according to ICH validation.
- It was successfully applied to the analysis of quaternary mixture in dosage form.

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