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ORIGINAL ARTICLE

Development and validation of two chromatographic methods for the simultaneous determination of raubasine and almitrine besmesylate in pharmaceutical dosage form

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Abstract A binary mixture of almitrine besmesylate (A) and raubasine (R) was determined by two different chromatographic methods. The first method was based on HPTLC separation of the two drugs followed by densitometric measurement of their spots at 245 and 285 nm for A and R, respectively. The separation was carried out using HPTLC silica gel F254 nanoplates with methanol:ammonia (10:8, v/v) as developing solvent. The linearity was achieved over concentration range of 0.5–8 μg/spot and 0.5–10 μg/spot with mean accuracy 100.79 ± 1.58 and 100.68 ± 1.78, for A and R, respectively. The second method involved the determination of A and R using reversed phase high performance liquid chromatography (HPLC) on C_{18} column using acetonitrile:potassium dihydrogen orthophosphate buffer pH = 4.7 (70:30, v/v) as mobile phase with flow rate at 2 ml/min. Quantitation was achieved using UV detection at 220 nm. A linear relationship was obtained over a concentration range of 0.75–105 μg ml^{-1} for both drugs with mean accuracy 100.85 ± 1.74 and 98.82 ± 1.31, for A and R, respectively. The methods were successfully applied for the determination of the cited drugs in dosage forms. The proposed methods were validated according to USP and were found to be valid and suitable for the assay of the cited drugs in dosage forms in quality control laboratories.

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1. Introduction

Raubasine is an alkaloid used as a vasodilator; chemically, it is related to reserpine.

Almitrine has been used as a respiratory stimulant in acute respiratory failure. It is also used in combination with raubasine for mental function impairment for the elderly.

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Fixed dose combinations containing A and R are widely available in market for the medication and management of peripheral vascular disorder.[1]

They have the following structures as shown in (Fig. 1).[2]

They are determined individually or simultaneously using different analytical methods. The determination of R was done by spectrophotometry,[3] electrochemical methods,[4] gas chromatography[5] and HPLC.[6–8]

The determination of A was done by gas chromatography[9] and HPLC methods.[8,10] The binary mixture was determined simultaneously by spectrophotometry[11–13] and by HPLC.[8,14]

The aim of this work is to develop simple, accurate, rapid, precise and validated chromatographic methods suitable for routine analysis of this combination in dosage forms and quality control laboratories.

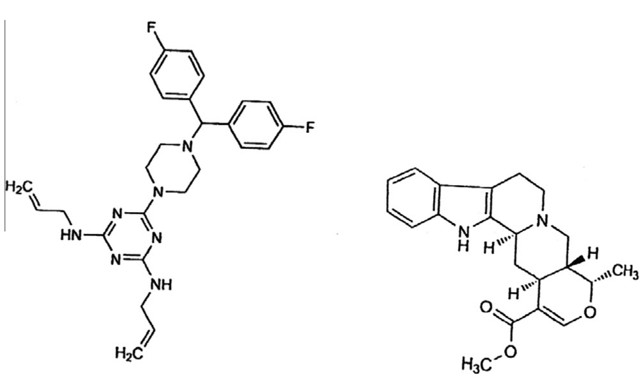
2. Experimental

2.1. Apparatus

1 Shimadzu TLC scanning densitometer CS 9301pc (Japan).
2 Precoated glass silica gel HPTLC nanoplates 10X10 GF254 (M N) (Germany).
3 Chromtech graduated glass micro syringe 25 µl (Taiwan).
4 Glass jar with lid (5X15X5).
5 HPLC chromatography; Agilent 1200 series equipped with Agilent quaternary pump G1311A, UV detector VWD G1314B and manual injector (20 µl loop) G1328B (Japan).
6 HPLC Column Thermo Hypersil BDS, C_{18} (250 × 4.6 mm) 5 µm.

2.2. Materials

- *Pure samples:* Raubasine and Almitrine besmesylate powders were kindly supplied by Servier Pharmaceutical Company, October City, Egypt. The purity was found to be 101.529 ± 1.65 and 97.77 ± 1.22 for A and R, respectively according to manufacturer procedure.



Alimitrine Besmesylate M.Wt=669.8 — Raubasine M.Wt=352.4

Figure 1 Chemical structures of A and R.

- *Market samples:* Duxil tablets (Servier, Egypt pharmaceutical company) with Batch number 11173 were purchased from the Egyptian market. Each tablet is claimed to contain 10 mg of raubasine and 30 mg of almitrine besmesylate.
- Triple distilled water – methanol for HPLC (SDFCL, India) – acetonitrile for HPLC (SDFCL, India) – methanol (AR) (SDFCL, India) – ammonia 33% (El NASR-EGYPT) – potassium dihydrogen orthophosphate (AR grade) (ADWIC, Egypt).
- Developing solvent for HPTLC method: methanol:ammonia (33%) (10:8, v/v).
- Mobile phase for HPLC: acetonitrile:potassium dihydrogen orthophosphate buffer (3.4 gm of KH_2PO_4 dissolved in 500 ml water) (70:30, v/v).

2.3. Preparations of standard solutions

2.3.1. Stock standard solutions

For HPTLC method. Solutions with a final concentration of 2 $mgml^{-1}$ in methanol were prepared for A and R.

For HPLC method. Two solutions were prepared for each of A and R with final concentrations 150 μgml^{-1} and 15 μgml^{-1} in methanol.

2.4. Laboratory prepared mixtures

HPTLC method

Into a series of 10- ml volumetric flask, different aliquots of stock standard solutions (2 mg ml^{-1}) were quantitatively transferred and the volume was completed to obtain final concentrations of 0.2, 0.6, 0.6, 0.2, 0.2 mg ml^{-1} and 0.6, 0.4, 0.2, 0.8, 0.2 mg ml^{-1} for A and R, respectively.

HPLC method

Into a series of 10- ml volumetric flasks, different aliquots of stock standard solutions were quantitatively transferred and the volume was completed to obtain final concentrations of 4, 6, 2, 9 µg m l^{-1} and 4, 4, 6, 3 µg m l^{-1} for A and R, respectively.

2.5. Pharmaceutical dosage form

10 tablets were accurately weighed and powdered. A quantity of powdered tablets equivalent to 10 mg R and 30 mg A was weighed into a 250- ml beaker and 50- ml methanol was added. The suspension was sonicated for 15 min then filtered into a 100- ml volumetric flask. The residue was washed three times each with 10- ml methanol and the washings were collected on the same 100- ml volumetric flask (solution A).

3- ml of solution A was quantitatively transferred into a 100- ml volumetric flask and the volume was completed with methanol (solution B).

3. Procedures

3.1. Construction of calibration curves

3.1.1. Working standard solutions

HPTLC method. Into 2 separate sets of 10- ml volumetric flasks, different aliquots of stock standard solutions

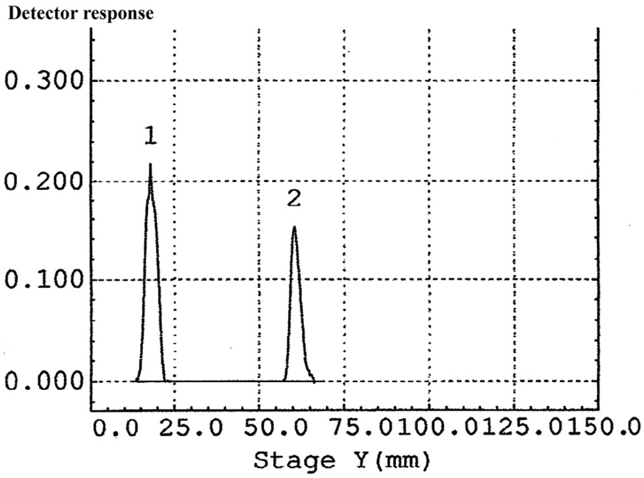


Figure 2 TLC chromatogram of mixture of (1) R 2 mg/ml and (2) A 1 mg/ml at 280 nm.

(2 mg ml^{-1}) were quantitatively transferred. The volume was completed with methanol to obtain final concentrations ranges of 0.1–1.8 mg m l^{-1} and 0.1–2 mg ml^{-1} for A and R, respectively.

5 µl from each of the working standard solutions was applied separately onto the HPTLC plate in triplicate. The spots were spaced 1 cm apart from each other and 1 cm from the bottom edge of the plate. The plate was developed ascendingly to a distance of 8 cm. using methanol:ammonia (10:8, v/v) as developing solvent in glass chamber previously saturated with developing solvent for 10 min at room temperature. The plate was removed, dried in air then scanned at 285 nm and 245 nm for R and A, respectively. The peak area was recorded. The calibration curve was plotted between peak area and concentration and the regression equation was computed.

HPLC method. Into two separate sets of 10- ml volumetric flasks, different aliquots of stock standard solutions of 150 µg ml^{-1} or 15 µg ml^{-1} were quantitatively transferred. The volume was completed with methanol to obtain a final concentration range of 0.75–105 µg ml^{-1} for both A and R.

20 µl from each of the working standard solutions was injected separately into the HPLC chromatograph, the flow rate was kept at 2 ml/min at ambient temp and eluent was monitored at 220 nm .The separation was performed on C_{18} column using acetonitrile:potassium dihydrogen orthophosphate buffer pH = 4.7 (70:30, v/v) as mobile phase. The peak area was recorded. The calibration curve was plotted between peak area and concentration and the regression equation was computed.

3.2. Laboratory prepared mixtures

HPTLC method: 5 µl of laboratory prepared mixture was applied onto TLC plate, the procedure described under construction of calibration curve was repeated and the concentration of each drug was computed from the regression equation.

HPLC method: 20 µl of laboratory prepared mixtures was injected separately into the HPLC chromatograph, the procedure described under construction of calibration curve was repeated and the concentration of each drug was computed from the regression equation.

3.3. Analysis of pharmaceutical dosage form

Proceed as detailed under Section 2.5 using 5 µl of test solution A for the HPTLC method and 20 µl of test solution B for HPLC method.

4. Results and discussion

By reviewing the literature in hand, it was found that no TLC methods were published for the simultaneous determination of binary mixture of A and R while few HPLC methods were reported[8,14]

Therefore the aim of this work was to develop and validate chromatographic methods for simultaneous determination of the cited drugs.

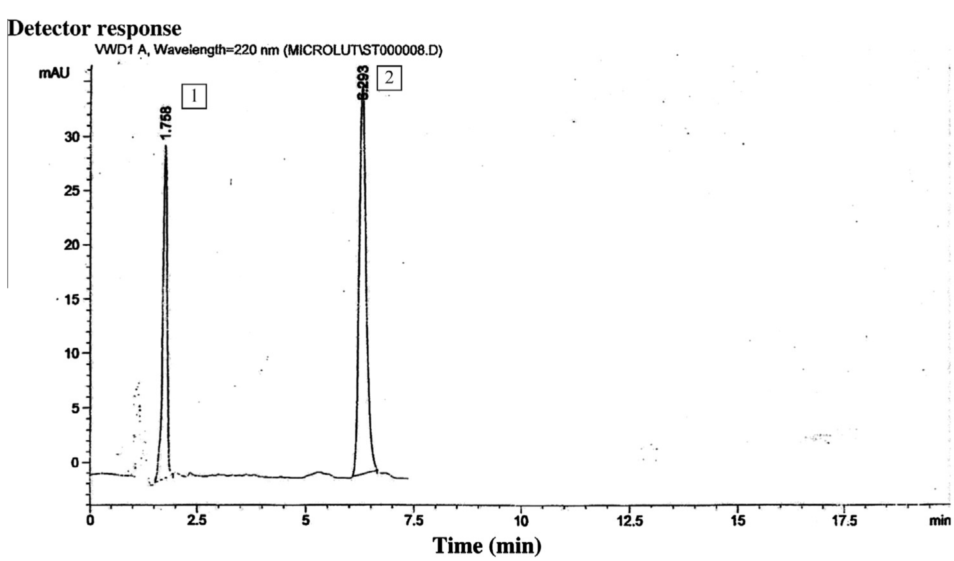


Figure 3 HPLC chromatogram of 20 µl injection of mixture of (1) R 10 µg/ml and (2) A 30 µg/ml.

Table 1 System suitability parameters for the proposed methods.

Parameter	HPTLC		HPLC	
	A	*R*	*A*	*R*
Retention time, min (R_t) or (R_f)	0.2	0.8	6.293	1.758
Tailing factor (*T*)	1	1	1	0.937
Theoretical plates (*N*)	–	–	2438.48	428.043
Capacity factor	4	0.25	2.52	4.79
Resolution	5.8		10.065	
Height equivalent to theoretical plate (HETP)	–	–	0.01	0.058

Table 2 Assay validation scheme and regression equation parameters for the proposed HPLC and HPTLC methods.

Parameter	HPTLC		HPLC	
	A	*R*	*A*	*R*
Concentration range	0.5–8 μg/spot	0.5–10 μg/spot	0.75–105 μg/ml	0.75–105 μg/ml
Regression equation				
-Slope	578.57	713.96	64.898	125.27
-SE of slope	25.92	7.53	0.883	0.54
-Intercept	694.01	640.48	-11.303	-82.423
-SE of intercept	5.548	27.27	15.07	25.86
-Correlation coefficient (*r*)	0.9998	0.9995	0.9998	0.999
Accuracy (Mean ± SD)	100.79 ± 1.58	100.68 ± 1.78	100.85 ± 1.74	98.82 ± 1.31
Precision				
-Intraday precision*	100.29 ± 1.82	99.24 ± 0.84	98.77 ± 1.87	98.21 ± 0.88
-Intermediate precision**	101.14 ± 0.42	99.61 ± 1.004	102.32 ± 0.38	101.62 ± 1.16
Robustness***	102.61 ± 0.21	99.49 ± 0.31	99.63 ± 0.15	98.57 ± 0.29
LOD# μg/ml or μg/ spot	0.0298	0.01636	0.153	0.0819
LOQ# μg/ml or μg/ spot	0.0995	0.0545	0.513	0.273

* For concentrations 3, 4, 5 μg/spot of *A* and 2.5,3,4 of *R* for HPTLC and 0.75,45,75 μg/ml of *A* and 15, 45, 75 μg/ml of *R* for HPLC.
** For concentrations 3, 4 μg/spot of *A* and *R* for HPTLC and 45, 75 μg/ml of *A* and 15, 45 μg/ml of *R* for HPLC and.
*** By changing pH ± 0.1, changing mobile phase composition, changing saturation time ± 5 min and changing the scaling wavelength ± 1 nm.
# Calculated according to the following equations LOD = 3SD/a and LOQ = 10SD/a, *a* = slop.

Table 3 Determination of *A* and *R* in laboratory prepared mixtures by HPTLC and HPLC methods.

	HPTLC					HPLC				
	A conc μg/spot	*R* conc μg/spot	Ratio	Recovery%		*A* conc μg/ml	*R* conc μg/ml	Ratio	Recovery%	
				A	*R*				*A*	*R*
	1	3	1:3	99.49	100.73	4	4	1:1	102.74	99.02
	3	2	3:2	101.66	101.8	6	4	3:2	99.50	101.37
	3	1	3:1	102.9	99.64	2	6	1:3	101.72	98.89
	1	4	1:4	98.25	101.28	9	3	3:1	99.80	102.74
	1	1	1:1	100.00	99.00					
Mean ± SD				100.46 ± 1.83	100.49 ± 1.15	100.49 ± 1.16			100.94 ± 1.55	100.50 ± 1.87

HPTLC method: to optimize TLC parameters, several developing solvents were tried as methanol:13.5 M ammonia (10:2, v/v), methanol:ammonia (33%) (10:10, v/v), methanol:ammonia (33%) (10:7, v/v). But the best resolution was achieved using developing solvent consisting of methanol:ammonia (33%) (10:8, v/v). Well defined spots were obtained when the chamber was saturated with developing solvent for 10 min at room temperature. The R_f values were found to be 0.2 and 0.8 for A and R, respectively. The wavelengths chosen were 245 nm and 285 nm for A and R, respectively which are the maximum wavelengths for the studied drugs to increase the sensitivity of the method. When the plate was scanned with a densitometer, sharp and symmetric peaks of A and R were obtained (Fig. 2) which allow the determination of both drugs with good accuracy and precision.

HPLC method: a simple method was adopted for the simultaneous determination of A and R either in bulk powder or in pharmaceutical dosage form. Different mobile phases were

Table 4 Application of the standard addition technique to the analysis of *A* and *R* in their dosage forms by proposed methods.

Dosage form	Found *%		Pure added (mg/ml)		Found * (mg/ml)		Recovery %	
	HPTLC	HPLC	HPTLC	HPLC	HPTLC	HPLC	HPTLC	HPLC
A in Duxil® 30.0 mg	101.1 ± 0.72	101.25 ± 0.39	1.5	3.0	1.548	3.037	103.2	101.23
of tablets (Batch no. 12353)			3.0	6.0	3.057	5.972	101.9	99.543
			3.0	12.0	2.959	11.7516	98.66	97.93
Mean ± S.D							101.58 ± 1.79	99.56 ± 1.65
R in Duxil® 10.0 mg	100.68 ± 0.79	101.25 ± 0.59	0.5	1.00	0.506	1.026	101.2	102.6
of tablets (Batch no. 12353)			1.00	2.00	0.9902	1.987	99.02	99.35
			1.00	4.00	0.984	3.968	101.62	99.2
Mean ± S.D							100.615 ± 1.39	100.38 ± 1.919

* Average of three determinations.

Table 5 Statistical comparison between proposed methods and manufacturer's method.

	HPTLC		HPLC		Manufacturer's method**	
	A	*R*	*A*	*R*	*A*	*R*
Bulk powder						
Mean accuracy	100.79	100.68	100.85	98.82	101.529	97.77
SD	1.58	1.78	1.74	1.31	1.65	1.22
Variance	2.496	3.168	3.027	1.716	2.74	1.48
n	6	6	6	6	6	6
F test	1.097	2.129	1.101	1.153		
t test	0.487	2.165	0.4073	1.135		
DF						
Mean accuracy	101.1	100.68	99.56	100.38	101.55	100.99
SD	0.72	0.79	1.65	1.919	0.74	0.62
Variance	0.5184	0.62	2.72	3.68	0.55	0.39
n	6	6	6	6	6	6
F test (4.95)*	1.07	1.585	4.90	2.64		
t test (2.228)*	1.454	1.062	1.334	0.664		

* The figures in parenthesis are the corresponding tabulated values at $P = 0.05$[16].
** UV Spectrophotometric method, Servier Egypt for bulk powder and HPLC method, Servier Egypt for dosage form, through personal communication.

tried as methanol (100%), methanol:water (85:15, v/v), methanol:water (90:10, v/v), methanol:potassium dihydrogen orthophosphate buffer (85:15, v/v), acetonitrile:buffer (80:20, v/v) different pH values were also tried but the best resolution was achieved using a mobile phase consisting of acetonitrile:potassium dihydrogen orthophosphate buffer pH = 4.7 (70:30, v/v) which gave good resolution and sensitivity of both drugs (Fig. 3).

The system suitability parameters were calculated according to the USP[15] and the values obtained are shown in Table 1.

Compared to reported HPLC methods, the proposed method has the advantages of being more economical than the manufacturer's method because the latter used methane sulphonic acid as solvent and heptane sulphonic acid in mobile phase which are expensive. The proposed HPLC method is also more sensitive and a more rapid method than the method of El-Sayed.[14] Furthermore, it was validated according to USP guidelines whereas the method of Wang et al[8] did not apply any validation scheme

The proposed methods were subjected to USP validation protocol[15] and the results obtained are shown in Table 2. The results in this table show that the method is reproducible and precise as shown by the small values of the RSD of the intraday and intermediate precision. The robustness of the method was studied by applying small and deliberate changes in the chromatographic conditions such as by changing pH ±0.1, changing mobile phase composition, changing saturation time ±5 min and changing the scaling wavelength ±1 nm. Low value of %RSD shows that the method is robust and that deliberate small changes in the studied factors do not lead to significant changes in R_t or R_f values, area or symmetry of the peaks.

The proposed methods were successfully applied for the determination of A and R and simultaneously analysed in the prepared mixtures with mean percentage recoveries of 100.46 ± 1.83 and 100.49 ± 1.15 for A and R, respectively by HPTLC and 100.94 ± 1.55 and 100.50 ± 1.87 for A and R, respectively by the HPLC method as shown in Table 3.

The proposed methods were successfully applied for the determination of A and R in D.F and the mean recovery obtained was 101.1 ± 0.72 and 100.68 ± 0.79 for A and R, respectively by HPTLC and 101.25 ± 0.39 and 101.25 ± 0.59 for A and R, respectively by HPLC as shown in Table 4.

The validation of the proposed methods was ascertained by application of the standard addition technique and the mean recoveries of added standard were 101.58 ± 1.79 and 100.615 ± 1.39 for A and R, respectively by HPTLC and 99.56 ± 1.65 and 100.55 ± 1.95 for A and R, respectively by HPLC as shown in Table 4.

Statistical comparison between the results of determination of A and R in D.F and in pure powdered form by the proposed methods and those of the manufacturer's method was done and no significant difference was observed at 95% confidence level as shown in Table 5.

5. Summary and conclusion

From the previous discussion, it could be concluded that the proposed methods are simple and do not require sophisticated techniques or instruments. The proposed HPTLC method is the first reported TLC method for the determination of the mixture. It has the advantage of allowing determination of several samples at the same time.

Both methods are also sensitive, selective and can be used for the routine analysis of raubasine, and almitrine besmesylate in their available dosage forms. The methods are also suitable and valid for application in quality control laboratories.

6. Conflict of interest

None.

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