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Stability-indicating high performance liquid chromatographic determination of raubasine in its binary mixture with kinetic study of raubasine acid degradation

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ABSTRACT

Stability-indicative determination of raubasine (RAB) in the presence of its degradate and its binary mixture with almitrine dismesylate (ALM) was investigated. The degradation product had been isolated, via acid-degradation, characterized and confirmed. Selective quantification of RAB and ALM in bulk form, pharmaceutical formulations and/or in the presence of RAB degradate was demonstrated. The analytical technique adopted for quantification was high performance liquid chromatography (HPLC). Separation was performed using a ZORBAX ODS column with a mobile phase consisting of acetonitrile + phosphate buffer pH 3.4 80:20 (v/v) with UV detection at 254 nm. The method showed high sensitivity with good linearity over the concentration range of 5–120 and 5–60 $\mu g\,mL^{-1}$ for RAB and ALM respectively. The HPLC method was used to study the kinetics of RAB acid degradation that was found to follow a first-order reaction. The activation energy could be estimated from the Arrhenius plot and it was found to be 18.152 kcal mol^{-1}.

1. Introduction

Raubasine (RAB) has the IUPAC name, [(19α)-16, 17 didehydro-19-methyl-oxayohimban-16-carboxylic acid methyl ester] (Fig. 1) [1]. It is an alkaloid obtained from *Rauwolfia serpentina*. It has been described as a vasodilator related chemically to reserpine [2].

The determination of RAB was studied by spectrophotometric [3–6], electrochemical [7], gas chromatographic [8] and high performance liquid chromatographic methods [9–16].

Almitrine dismesylate (ALM) has the IUPAC name, 6-[4-[Bis (4-fluorophenyl) methyl]-1-piperazinyl] -N, N′-di-2-propenyl-1, 3, 5-triazine-2, 4-diamine (Fig. 2) [1]. It has been used as a respiratory stimulant in acute respiratory failure. Also, it is used in a combination with raubasine for mental function impairment for the elderly [2].

The determination of ALM was studied by spectrophotometric [4–6], several gas chromatographic [17–19] and high performance liquid chromatographic methods [12,20,21].

In modern analytical laboratory, there is always a need for significant stability-indicating methods of analysis. The focus of the present study was to develop and validate simple stability-indicating method for the quantification of RAB and ALM in bulk form or in the presence of RAB acid-degradate. Moreover, kinetic studies and accelerated stability experiments are important to solve problems encountered in quality control and to predict the expiry dates of pharmaceutical products.

The scientific novelty of the present work is that the suggested method represents the first stability indicating HPLC method for the analysis of this mixture. Besides, it is the first kinetic study for RAB degradation to calculate the strength of this ester molecule. In addition that, this kinetic study is monitored by HPLC technique, which is more accurate, indicative and specific than the ordinary UV spectroscopy.

2. Experimental

2.1. Instruments

- Precoated HPTLC plates, silica gel 60 F_{245} 20 cm × 20 cm, 0.2 nm thickness, Macheray-Nagel (Germany).
- Gas chromatograph coupled to a mass spectrometer (GC–MS): SHINADZU GC-MS-QP 1000 EX, Finnigan Nat (USA) composed of gas chromatographer (GC-14A) and mass spectrometer of electron voltage (70 eV). The GC–MS conditions were: column; polyethylene glycol (Atwax), mobile phase; helium gas. Temperature program: initial temp: 120 °C, initial time: 1 min, program rate: 10 °C min^{-1}, final temp: 210 °C. Injector: 250 μL, detector temperature: 250 °C.
- A liquid chromatograph consisted of an "Agilent" HPLC instrument, isocratic pump (Model G 1310 A pump, Agilent 1100 series),

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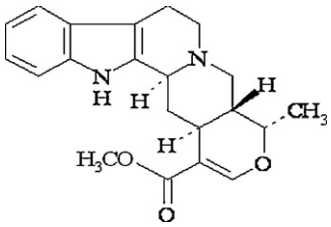

Fig. 1. The structure of raubasine.

connected with an UV detector (Model G1314 A, Agilent 1100 series). The injector was a manual Rheodyne injector (Model 7725I, Rohnert Park, CA, USA) equipped with a 20 μL injector loop, Agilent (USA). The instrument was connected to an IBM compatible PC and an HP diskjet 5652 printer.

The chromatographic conditions were: Stationary phase: a 250 mm × 4.6 mm i.d. C_{18} ZORBAX, 5 μm analytical column. Mobile phase: acetonitrile + solution pH 3.4 (Dissolve 1.74 g of dipotassium hydrogen phosphate anhydrous and dilute to a volume of 900 mL with deionised water, add 1 mL triethylamine and 0.1% sodium heptane sulphonate and adjust to a pH of 3.4 with 85% phosphoric acid) 80:20 (v/v). The mobile phase was filtered through 0.45 μm Millipore membrane filter and was degassed for 30 min in an ultrasonic bath prior to use. UV detection was done at 254 nm. The system was operated at ambient temperature. The flow rate was isocratic at 1.0 mL min^{-1}. The samples were filtered also through a 0.45 μm membrane filter, and were injected by the aid of a 25 μL Hamilton® analytical syringe.

2.2. Materials and reagents

Reference RAB, molecular weight 352.4 [CAS number 483-04-5] and ALM molecular weight 669.8 [CAS number 27469-53-0] powder were kindly supplied by Servier, Egypt Their purity were checked in our laboratory according to the manufacturer's method (HPLC method using C18 as a stationary phase and methanol:distilled water:sodium heptane sulfonate 5.5% [85:13:2 v/v/v] as a mobile phase) and were found to be 100.23 ± 1.247 and 99.89 ± 1.023 for RAB and ALM respectively.

Pharmaceutical dosage form (Duxil tablets) batch numbers 13753, 11424 (exp. 11/09) were purchased from the Egyptian market. Each tablet is claimed to contain 10 mg of RAB and 30 mg of ALM. Duxil tablets are manufactured by Servier, Egypt pharmaceutical company.

All chemicals used were of analytical grade and de-ionized water was HPLC grade. Hydrochloric acid, methanol, chloroform, ethyl acetate, acetonitrile for HPLC, dipotassium hydrogen phosphate anhydrous, sodium heptane sulphonate, phosphoric acid and triethylamine were obtained from Merck (Germany).

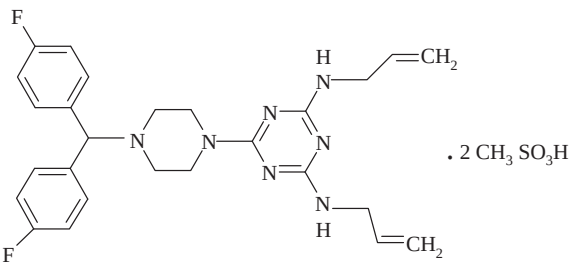

Fig. 2. The structure of almitrine dismesylate.

2.3. Standard solutions

RAB stock solution (0.25 mg mL^{-1}) in acetonitrile. ALM stock solution (0.25 mg mL^{-1}) in acetonitrile. RAB degradation product stock solution (0.25 mg mL^{-1}) in acetonitrile.

2.4. Procedures

2.4.1. Degradation of raubasine [6]

Accelerated acid degradation was performed. 0.2 g of pure RAB was accurately weighed and dissolved in 100 mL of 2 mol L^{-1} hydrochloric acid (HCl). The solution was refluxed at 100 °C. Complete hydrolysis was obtained after 6.5 h as investigated by thin layer chromatography (TLC). The solution was evaporated near dryness.

The degraded solution was applied as a band to TLC plates. The plates were placed in a chromatographic tank previously saturated for 1 h with the mobile phase methanol + chloroform + ethyl acetate 2:1:1 (v/v/v) and then air dried.

The band was visualized under UV light at 254 nm, then scraped and suspended in the least amount of methanol. The suspension was filtered and left to evaporate at room temperature (25 °C) to obtain the degradation product. The degradate powder was elucidated by UV spectroscopy [6] and GC/MS.

2.4.2. Linearity

Portions 0.5–12.0 mL from RAB stock solution (0.25 mg mL^{-1}) and 0.5–6.0 mL from ALM stock solution (0.25 mg mL^{-1}) were transferred separately into a series of 25 mL measuring flask then completed to volume with acetonitrile. 25 μL of the previous solutions were injected into the liquid chromatograph using the chromatographic conditions described under Section 2.1. The corresponding peak areas were measured and calibration curves representing the ratio of the relative peak area of RAB and ALM to that of the external standard (20 μg mL^{-1} of RAB and 10 μg mL^{-1} ALM) were constructed, vs. the corresponding concentrations of RAB and ALM in μg mL^{-1}.

2.5. Kinetic studies

2.5.1. For studying the kinetic order of the reaction

Into a 100 mL measuring flask, 0.2 g of RAB in 2 mol L^{-1} hydrochloric acid (HCl) were dissolved and completed to the mark with the same solvent. This solution was transferred into another clean dry conical flask and refluxed in a thermostatically controlled water bath at 80 °C for 6.5 h. 1.5 mL sample solutions were taken at 2.5 h then at 1 h intervals, placed into 25 mL measuring flasks half filled with cold acetonitrile, neutralized with 1.5 mL 2 mol L^{-1} sodium hydroxide, and the volume was completed with cold acetonitrile. The solutions (initial concentration C_0 = 120 μg mL^{-1}) were injected in the liquid chromatograph using the chromatographic conditions described above. The concentration of RAB was calculated from the regression equation. The log % of remaining concentration against time was plotted.

2.5.2. For studying the effect of HCl concentration on the reaction rate

Into a series of 100 mL measuring flasks, 0.2 g of RAB were dissolved in 1.0, 1.5, and 2 mol L^{-1} HCl and completed to the mark with the same solvent. These solutions were transferred into other clean dry conical flasks, and then refluxed in a thermostatically controlled water bath at 80 °C for 6.5 h. 1.5 mL sample solutions were taken at 2.5 h then at 1 h intervals and then completed as described under Section 2.5.1. The log % of remaining concentration against

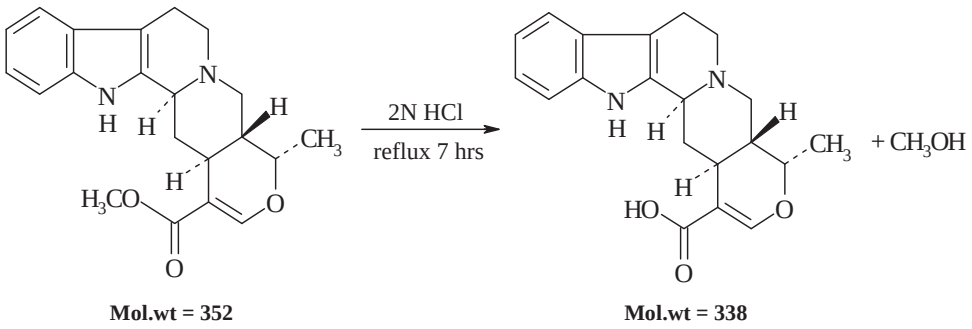

Fig. 3. The proposed scheme for the degradation of raubasine.

time was constructed for different molarities of HCl and the rates constant and $t_{1/2}$ were calculated.

2.5.3. For studying the effect of the temperature on the reaction rate

Three portions each of 0.2 g of RAB were dissolved in 100 mL measuring flasks and completed to volume with 1.0, 1.5, and 2 mol L^{-1} HCl respectively. These solutions were transferred into other clean dry conical flasks and then refluxed in a thermostatically controlled water bath at 60 °C, 70 °C, 80 °C and 90 °C for 6.5 h. 1.5 mL sample solutions were taken at 2.5 h then at 1 h intervals and then completed as described under Section 2.5.1. The log % of remaining concentration against time at different temperatures was plotted. Also the Arrhenius plot for the effect of temperature on the rate of hydrolysis was constructed.

3. Results and discussion

3.1. Degradation of raubasine

Degradation was obtained through two major pathways, via hydrolysis by acids, bases, moisture or via oxidation by light and/or hydrogen peroxide. Being an ester, RAB was expected to be hydrolyzed. On the contrary, ALM had no ester or amide group so it resists hydrolysis, also it did not have an oxidisable group. So due to bad storage, only RAB was expected to be degraded by hydrolysis.

The International Conference on Harmonization (ICH) guideline entitled "Stability testing of new drugs substances and products" requires the stress testing to be carried out to elucidate the inherent stability characteristics of the active substance [22]. An ideal stability indicating method is one that quantifies the standard drug alone and also resolves its degradation products.

The proposed scheme for degradation of RAB was shown in Fig. 3. The GC/MS could verify the degradation product. The appearance of one peak in the GC/MS chart indicated its purity because the presence of impurities would increase the number of peaks in the GC chart. In the MS chart, the parent peak was identified at m/z = 338 indicating the molecular weight of the degradation product as z (the charge) was one.

Using the NMR spectra to identify the structure of the degradation product was useless as no change in the type and number of proton signal occurs. Moreover, in the IR spectra, no change occurs in the functional groups except for the new C=O and O–H groups of the carboxylic acid in the degradation product. The C=O band will appear in the double bond region (1600–1800 cm^{-1}) and thus it will be somewhat masked by the C=C of the aromatic benzene ring and the O–H band will appear in the region (3000–3300 cm^{-1}) will be masked by N–H in the RAB molecule, i.e., the IR spectra will be unvalued [23].

TLC-monitoring of the drug degradation was done on thin layer plates using methanol + chloroform + ethyl acetate 2:1:1 (v/v/v) as a developing system [24]. The developed plates were visualized under short UV lamp (R_f value = 0.6, 0.87 and 0.44 for RAB, ALM and RAB degradate respectively).

3.2. High performance liquid chromatographic analysis

A simple isocratic high-performance liquid chromatographic method was developed for the determination of RAB and ALM in the presence of RAB degradation product without prior separation. To optimize the HPLC assay parameters, type of column and its dimensions, mobile phase conditions, and choice of detection wavelength were investigated. Different types of stationary phase C_8 and a ZORBAX ODS column with different dimensions and par-

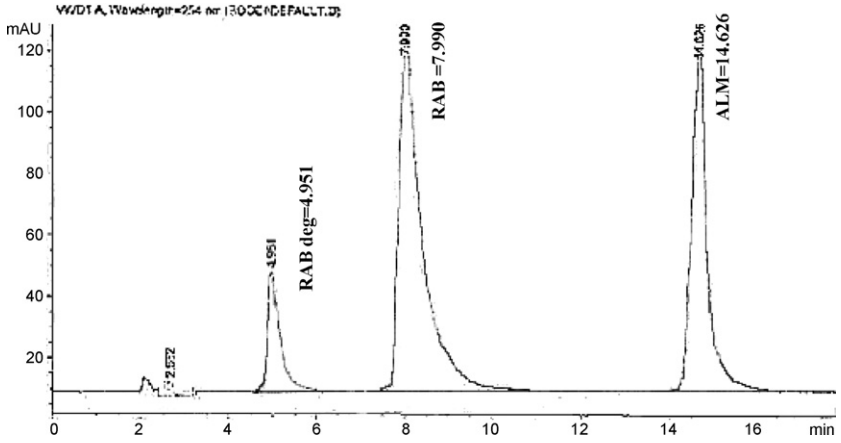

Fig. 4. Liquid chromatographic separation of RAB (120 μg mL^{-1}), ALM (60 μg mL^{-1}) and RAB degradation product (30 μg mL^{-1}) using the chromatographic conditions described in the text.

Table 1
Parameters of system suitability test of HPLC method.

Parameter	Obtained value		Reference value
	Raubasine	Almitrine dismesylate	
Resolution (R)	2.885	$R > 0.8$	
T (tailing factor)	1.15	1.0	$T = 1$ for a typical symmetric peak
((relative retention)	2.243	>1	
k' (column capacity)	2.01	4.51	1–10 acceptable
N (column efficiency)	177.33	707.17	Increases with efficiency of the separation
HETP	0.14	0.035	The smaller the value, the higher the column efficiency

ticle size were used. It was found that the ZORBAX ODS column (250 mm × 4.6 mm i.d.) with a particle size of 5 μm gave the most suitable resolution. The mobile phase was chosen after several trials to reach the optimum stationary/mobile-phase matching. The peak shape improved dramatically by decreasing the percentage of buffer to acetonitrile in the mobile phase [24]. Tailing of the drug peak was reduced by the addition of triethyl amine. Optimum separation was obtained with a mobile phase consisting of acetonitrile: solution pH 3.4 added to it 1 mL triethylamine and 0.1% sodium heptane sulphonate 80:20 (v/v) with a retention time of 7.99 ± 0.02 min for RAB, 14.62 ± 0.2 min for ALM and 4.96 ± 0.03 min for RAB degradation product. (Fig. 4). System suitability parameters were tested by calculating the capacity factor, tailing factor, the sensitivity factor, column efficiency, and selectivity factor (resolution) [25–28] (Table 1). The chromatographic system described in this work allowed complete base line separation of RAB from its degradation product and ALM.

The suggested HPLC method, Wang's HPLC method [12] which is also the company method is fast (retention time of raubasine is 1 min, and almitrine is 4 min) but our method offered advantages of being stability indicating and has better resolution after calculating the system suitability parameters.

Linear relationships were obtained between the relative peak area at the selected wavelength 254 nm and the corresponding concentrations in the range of 5–120 μg mL^{-1} for RAB and 5–60 μg mL^{-1} for ALM, by adopting the external standard method for calibration.

The regression equations were computed and found to be:

$$A_1 = 0.0489\,C_1 + 0.0213 \quad r = 0.9996$$

$$A_2 = 0.0988\,C_2 - 0.0063 \quad r = 0.9997$$

where A_1 and A_2 are the relative peak areas to that of the external standard, C_1 and C_2 are the concentration in μg mL^{-1} for raubasine and almitrine dismesylate respectively and r is the correlation coefficient.

3.2.1. HPLC validation

The selectivity and specificity of the proposed method was proved by the analysis of laboratory prepared mixtures containing different ratios of the selected analytes (Table 2).

To ascertain the accuracy of the proposed procedure, it was successfully applied for the determination of RAB and ALM in Duxil tablets (Table 3). It is clear from the table that the company method failed to quantify raubasine in the expired Duxil batch. The validity was assessed by applying the standard addition technique. The small relative standard deviations indicate that the method is accurate (Table 4).

The results obtained for the analysis of RAB and ALM in the pure powdered form were statistically compared with those from a previously reported method [12]. A significant difference was not observed [29] (Table 5).

The precision of the suggested method was also expressed in terms of relative standard deviation of the inter-day and intra-day analysis. The robustness of the HPLC method was investigated by analysis of samples under a variety of experimental conditions such as small changes in the pH (3.0–3.5), and changing the column using a 250 mm × 4.6 mm i.d. C_{18} Lichrosorb 10 μm analytical column. The effect on retention time and peak parameters was studied. It was found that the method was robust when the column and the mobile phase pH were varied. During these investigations, the retention times were modified, however the areas and peaks symmetry were conserved. Changes in instruments or personnel did not alter the results, which indicate the ruggedness of the proposed method. The obtained assay parameters and a validation sheet [25] are presented in Table 6.

3.3. Kinetics of the degradation

The linear relationship (Fig. 5) between the log % of remaining concentration against time indicated first-order degradation. Since the hydrolysis was performed in a large excess of HCl (2.0 mol L^{-1}), therefore it follows a pseudo-first order reaction rate [30] which is the term used when two reactants are involved in the reaction but one of them is in such a large excess (HCl) that any change in its concentration is negligible compared with the change in concentration of the other reactant (drug).

Table 2
Determination of raubasine and almitrine in laboratory prepared mixtures by the proposed HPLC method.

Concentration (μg mL^{-1})			Ratio	HPLC method	
RAB	ALM	RAB deg	RAB:ALM:RAB deg	Recovery %	Recovery %
				RAB	ALM
20	40	5	4:8:1	99.53	99.14
10	25	5	2:5:1	100.55	101.85
10	30	Zero	1:3:zero	100.34	100.24
15	45	5	3:9:1	100.48	99.19
15	45	10	3:9:2	98.70	100.40
20	60	20	3:9:3	100.43	98.76
10	35	5	2:7:1	101.51	100.71
10	40	Zero	1:4:zero	98.37	102.53
Mean ± S.D.				99.98 ± 1.047	100.35 ± 1.335

Table 3
Determination of raubasine and almitrine in duxil tablets by the proposed methods.

Batch number	HPLC method		Reported method [a]	
	Found % ± S.D. of RAB[b]	Found % ± S.D. of ALM[b]	Found % ± S.D. of RAB[b]	Found % ± S.D. of ALM[b]
13753	97.90 ± 0.980	98.63 ± 1.273	98.95 ± 1.346	98.11 ± 1.445
11424 Exp.(11/09)	86.30 ± 1.175	97.82 ± 1.310	[c]	97.79 ± 1.502

[a] Manufacturer's HPLC method.
[b] Average of four determinations.
[c] Peak of RAB and its degradate are overlapped.

Table 4
Application of standard addition for the determination of raubasine and almitrine by the proposed methods.

Batch number	Standard added (mg)		HPLC method	
	RAB	ALM	Recovery % of added RAB	Recovery % of added ALM
13,753	25.00	25.00	99.32	99.21
	37.50	37.50	98.23	99.74
	50.00	50.00	100.40	101.21
Mean ± S.D.[a]			99.31 ± 1.085	100.05 ± 1.036

[a] Average of four determinations.

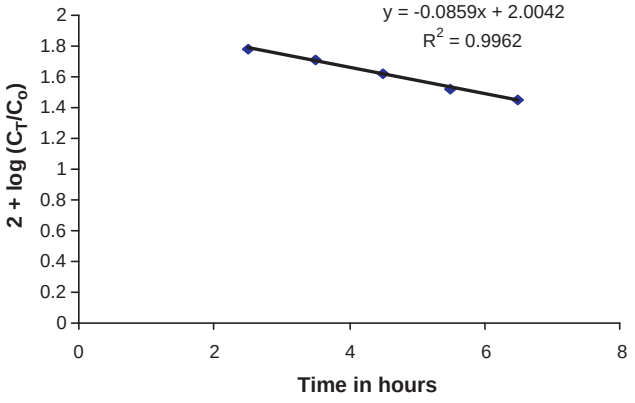

Fig. 5. First order plot of the hydrolysis of RAB (1000 mg %) with 2 mol L^{-1} HCl at 80 °C.

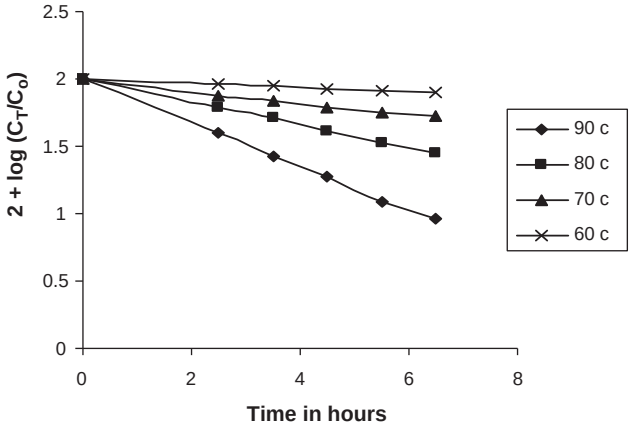

Fig. 6. First order plot of the hydrolysis of RAB (1000 mg %) with 2 mol L^{-1} HCl at different temperatures.

Different parameters that affect the rate of the reaction were studied. The temperature dependence of raubasine degradation was studied by conducting the reaction at different temperatures using different concentrations of the acid solution (Figs. 6–8). At each temperature the rate constant and $t_{1/2}$ were calculated then the log of the rate constant was plotted against the reciprocal of the temperature in Kelvin units (Arrhenius plot, Fig. 9) to demonstrate the effect of temperature on the rate constant. It was concluded that as the temperature increased the rate of hydrolysis increased with a decrease in the $t_{1/2}$ (Table 7). Also, the energy of activation was determined by calculating the rate constant from the following equation [31].

$$\log \frac{k_2}{k_1} = \frac{E_a}{2.303R}\left(\frac{T_2 - T_1}{T_1 T_2}\right)$$

where "E_a" is the activation energy, "T_1" and "T_2" are the two temperatures in Kelvin, "R" is the gas constant, and "k_1" and "k_2" are the rate constants at the two temperatures used.

The calculated "E_a" was found to be 18.152 kcal mol^{-1} which was a comparatively high value for esters, suggesting the stability in acid medium [32].

Another factor that affects the rate of the reaction is the acid strength of HCl, thus different normalities were used to study

Table 5
Statistical comparison for the results obtained by the proposed methods and the reported method.

Item	HPLC method		Reported method[a]	
	RAB	ALM	RAB	ALM
Mean	100.05	100.04	100.23	99.89
S.D.	0.945	1.085	1.247	1.023
Variance	0.893	1.177	1.555	1.046
N	8	7	6	6
F test	1.741 (3.69)	1.125 (4.39)		
Student's *t* test	0.308 (2.179)	0. 255 (2.201)		

The figures in parenthesis are the corresponding tabulated values at $P = 0.05$ [29].
[a] Manufacturer's HPLC method.

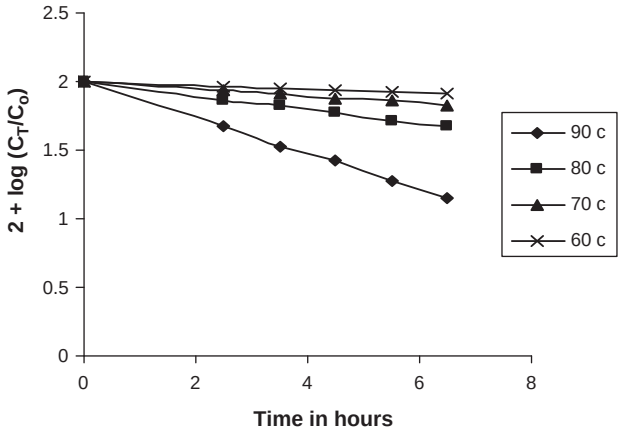

Fig. 7. First order plot of the hydrolysis RAB (1000 mg %) with 1.5 mol L^{-1} HCl at different temperatures.

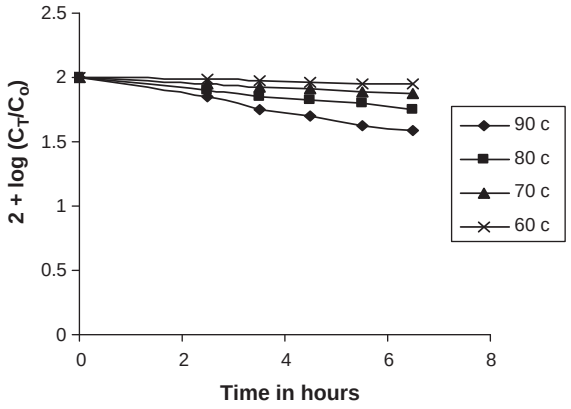

Fig. 8. First order plot of the hydrolysis of RAB (1000 mg %) with 1.0 mol L^{-1} HCl at different temperatures.

the hydrolysis reaction. The rate of hydrolysis increased with an increasing HCl concentration, although the effect was minor compared to the effect of temperature (Figs. 3–5 and Table 7).

In conclusion, the acid hydrolysis of raubasine was found to follow a pseudo first order reaction rate. Also the reaction rate increases with increase in the temperature and the strength of the acid solution.

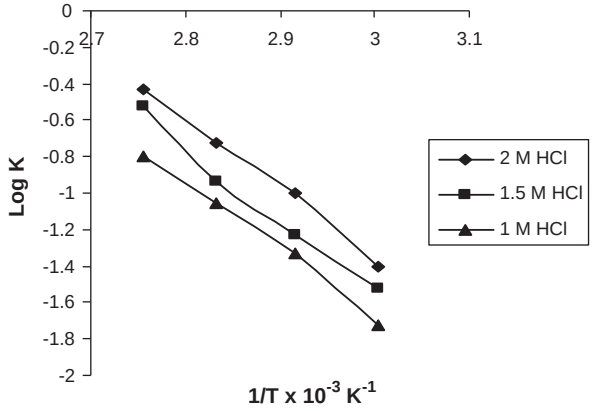

Fig. 9. Arrhenius Plot for the hydrolysis of RAB (1000 mg %) with 1.0, 1.5 and 2.0 mol L^{-1} HCl.

Table 6
Assay parameters and method validation sheet [25].

Parameter	HPLC method	
	RAB	ALM
Range (μg mL^{-1})	5.0–120.0	5.0–60.0
Slope	0.0489	0.0988
Intercept	0.0213	−0.0063
Mean	100.05	100.04
S.D.	0.945	1.085
Variance	0.893	1.177
Correlation coefficient (r)	0.9996	0.9997
Coefficient of variation	0.944	1.176
RSD %[a]	0.907, 1.173	0.660, 0.989
RSD %[b]	1.426, 1.338	1.581, 1.412
LOQ	3.5 μg mL^{-1}	

RSD %[a], RSD %[b] the intra-day, inter-day respectively (n = 5) relative standard deviation of concentrations (40 and 80 μg mL^{-1} of raubasine and 30 and 40 μg mL^{-1} of almitrine dismesylate) for the HPLC method.
LOQ: limit of quantification.

Table 7
Kinetic data of raubasine acid degradation.

Molarity of HCl	Temperature (°C)	k (h^{-1})	$t_{1/2}$ (h)
2.0 mol L^{-1} HCl	90	0.369	1.878
	80	0.190	3.647
	70	0.100	6.930
	60	0.039	17.769
1.5 mol L^{-1} HCl	90	0.301	2.302
	80	0.117	5.923
	70	0.059	11.745
	60	0.030	23.100
1.0 mol L^{-1} HCl	90	0.158	4.386
	80	0.089	7.786
	70	0.047	14.744
	60	0.019	36.473

4. Conclusion

The proposed HPLC method provides a simple, sensitive, and selective method suitable for the quality control analysis of RAB and ALM either in the pure powdered form or available pharmaceutical dosage forms with no interference from excipients or the degradation product.

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