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Validation of a simple method for simultaneous determination of lipoic acid and resveratrol by high performance liquid chromatography

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A high-performance liquid chromatography (HPLC) method was developed and validated for simultaneous determination of resveratrol (RSV) and lipoic acid (LA). A C18 column was used with a mobile phase consisting of acetonitrile and 0.01M phosphoric acid (60:40). The detection wavelength was at 235 nm. The method was specific in the presence of pharmaceutical excipients widely used in solid dosage forms or lipid-core nanocapsules. The results demonstrated linearity between 5 and 50 µg/mL for RSV and 30 and 120 µg/mL for LA. The method presented precision and accuracy (RSD <5%). In addition, the developed method was considered robust. Therefore, the developed method can be applied successfully for simultaneous determination of RSV and LA in the proposed conditions, with a potential application to assay both drugs in several dosage forms.

Keywords: Lipoic acid, resveratrol, HPLC, validation, simultaneous determination

Introduction

Antioxidants can decrease or inactivate free radicals and/or reduce oxidative damage (1). Polyphenols are a group of antioxidants widely used as treatment in many diseases. Resveratrol (RSV) (Figure 1a) is a natural polyphenol found mostly in grapes and wine (2). Recently, studies demonstrated that RSV has many biological activities, including cardioprotective (3), chemopreventing (4), neuroprotective (5), anti-inflammatory (6) and antioxidanteffects (7).

Similarly, Lipoic Acid (LA) (Figure 1b) is a potent antioxidant with reactive oxygen species scavenging activity, ability to repair other antioxidants (such as ascorbic acid, α-tocoferol and glutathione) in physiological and physiopathological *in vivo* conditions and can also chelate metals (8,9). LA can prevent sun damage aging, helping lesion recovery (10), preventing inflammation of the skin and a photocarcinogenesis caused by ultraviolet radiation (11). Thus, LA emerges as a potent antioxidant drug and also can be used in association with other antioxidants as it has the ability to interact with them and help their recovery (12).

The association of antioxidants with different mechanisms of action shows potential for use in a variety of pharmaceutical applications. This strategy aims a synergic effect from drugs. Also, the use of antioxidants in association with conventional therapies is an interesting approach, being able to increase drug effect and/or protect healthy cells from some drug's toxicity (13,14). The association of RSV and LA is promising because of RSV's pharmacological properties and LA's protection effects besides its capacity to interact with other antioxidants. It has been described that this association has neuroprotective effects, resulting from the effect of both drugs on multiple pathways (15), being of potential interest in combined therapy and association in pharmaceutical formulations.

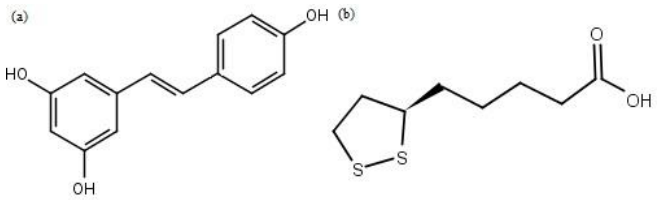

Figure 1. Chemical structures of (a) RSV and (b) LA.

A simultaneous analysis of two or more components in pharmaceutical dosage forms without prior chemical separation is an analytical challenge (16). In this way, a validation of analytical methods for simultaneous detection of drugs is a valuable tool to achieve reduction of costs and time. More than that, the validation is essential to produce reliable results between laboratories and for commercial reasons (17). The pharmaceutical potential of the association of LA

and RSV and the lack of a method for the simultaneous detection of these drugs motivated the present study, which aims to develop and to validate a high performance liquid chromatography analysis with ultraviolet detection (HLPC-UV) method for simultaneous determination of LA and RSV.

Experimental

Materials

The analytical grade reagents used were acetonitrile (ACN) from Merck and Pancreac and phosphoric acid from Merck. The RSV and LA were obtained from Fagron (Brazil) and Pharma Nostra (Brazil) respectively, with a purity of 99.20% e 99.95% respectively.

Instrumental

The RSV and LA were quantified by HPLC-UV. The equipment consisted of a PerkinElmer S-200 with an UV-vis detector (PerkinElmer, Shelton, Connecticut, USA). A Phenomenex Phenosphere-Next C18 column (150 mm x 4.6 mm, 5 μm part size, 1.1E+6 angström pore diameter) with a guard column were used in the experiments.

Chromatographic Conditions

The wavelength was determined from a UV spectrum from 200 to 500 nm in a Shimadzu UV-1800 Spectrophotometer, using RSV (0.01 mM) and LA (0.05 mM) reference solutions. For detection, a wavelength of 235nm was selected. The mobile phase (MP) consisted of ACN and 0.01M phosphoric acid (60:40), in a flow rate of 0.8 mL min. The choice of MP was made from a previous study with lipoic acid (Kulkamp et al. 2009).

RSV and LA solutions were diluted to give a final theoretical concentration of 1000 μg/mL. Thereafter, an aliquot was diluted in the MP. The injection volume was 20 μL.

Specificity

Specificity was evaluated in the presence of unloaded lipid core nanocapsules containing: caprylic capric triglycerides, sorbitan monostearate, poly (Ɛ-caprolactone) and acetone as components of organic phase and polysorbate 80 and ultrapure water as components of the aqueous phase, prepared by the method of precipitation of preformed polymers (18). For the evaluation of specificity, excipients traditionally used in solid dosage forms have also been used (lactose, magnesium stearate, silicon dioxide and starch) to prepare placebo solution. Solutions were prepared containing the unloaded lipid core nanocapsule, and a solution containing the mixture of the excipients: starch, magnesium stearate, lactose and mannitol. The solutions were diluted in MP and injected under the same method validation conditions. After the analysis, the chromatograms were compared.

Linearity

The analytical curves were obtained through a solution of LA and RSV in the concentration of 1000 μg/mL, and prepared using 5 concentrations of RSV and LA alone or in combination. The solutions containing both drugs were named RSV_{ass} and LA_{ass} (Table 1). All solutions were prepared in acetronitrile and diluted in the MP. The curves were prepared and analyzed on three different days. Linearity was determined through the linear regression, using the method of least squares, and the analysis of variance (ANOVA) was calculated, evaluating the statistical parameters. The range of the analytical curves was based on previous reported methods for each isolated substance (19,20).

Limits of detection and quantitation

The limits of detection and quantification were determined mathematically using the standard deviation of the value of the intercept with the Y axis and slope of three calibration curves (21,22). The limits of detection and quantification were evaluated based on the previously constructed calibration curve.

Precision

The experiments were evaluated with the drugs isolated or in combination. The tested concentrations were the central point of the

standard curves, which corresponds to the runs 3, 8 and 13 from Table 1.

The repeatability (intra-day precision) was evaluated by calculating the relative standard deviation (RSD) from each drug determination. The RSV and LA solutions were analyzed at concentrations of 25 μg/mL and 75 μg/mL, respectively, alone or in combination in nine successive determinations performed on the same day. Thus, the results are obtained with the same experimental conditions (method, equipment, day and analyst). The experimental results were expressed as RSD, with an acceptable value of ≤ 5.0%.

The intermediate precision (inter-day) of the method was performed at same concentrations described above. Three successive determinations were performed on three different days. The experimental results were considered acceptable when RSD ≤ 5.0%.

Accuracy

The concentrations of RSV and LA tested were 5, 25 and 50 μg/mL and 30, 75 and 120 μg/mL respectively. The association of the drugs was also evaluated in the same concentrations. The tested concentrations corresponds to the runs 1,3,5, 6, 8, 10, 11,13 and 15 from Table 1.

This parameter was determined by the recovery test that consisted on adding known amounts of reference substance to the placebo solution (prepared according to the specificity test described above). The samples were diluted in acetonitrile and centrifuged at 5000 rpm. An aliquot of the supernatant was then collected and diluted in the MP, and thus analyzed under the described conditions. The analyses were performed in triplicates. Accuracy was expressed as percentage of RSV and LA recovered in the lowest, intermediate and highest concentrations, with an acceptable RSD value of ≤ 5.0 %.

Robustness

The tested concentrations were the central point of the standard curves, which corresponds to the runs 3, 8 and 13 from Table 1. Solutions with concentrations of 25 μg/mL for RSV and 75 μg/mL for LA were prepared. Three replicates were performed on the same day with each sample, with small variations in the method. The variations were in the ACN supplier (ACN1 - Merck and ACN2 - Pancreac), in the MP flow (0.7 mL/min and 0.9 mL/min) and in the ACN/phosphoric acid proportion on the MP (65/35 and 55/55). The experimental results were expressed as RSD, with an acceptable value of ≤ 5.0 %.

Table 1. Concentration of RSV and LA analyzed by HPLC-UV, when tested isolated or in association.

Run Identification	Group	RSV (μg/mL)	LA (μg/mL)
1	RSV	5	-
2		15	-
3		25	-
4		40	-
5		50	-
6	LA	-	30
7		-	50
8		-	75
9		-	100
10		-	120
11	RSV_{ass} / LA_{ass}	5	30
12		15	50
13		25	75
14		40	100
15		50	120

Statistical analysis

The T test was performed for the paired comparisons and ANOVA one way for the other comparisons, using GraphPad Prism 5 software.

Results and Discussion

Validation of the quantification method

The development of the method aimed the simultaneous detection of RSV and LA in HPLC-UV. The UV spectra of the standard solutions of RSV (0.01 mM) and LA (0.05 mM) are shown in Figure 2. The wavelength of 235nm was selected for the simultaneous quantification of the compounds to avoid a possible interference from the excipients in lower wavelengths and baseline destabilization; and, at the same time, to allow the detection of both drugs.

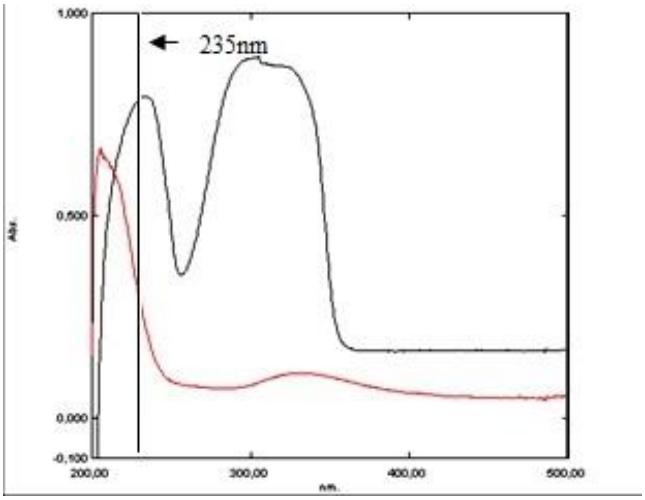

Figure 2. UV spectra of standard solutions: RSV 0.01mM (black line) e LA 0.05mM (red line).

The Figure 3 presents the chromatogram of both drugs, injected isolated or simultaneously. The concomitant analysis showed a separation with resolution of 2.28 and symmetry, with values close to 1, both values considered acceptable (21).

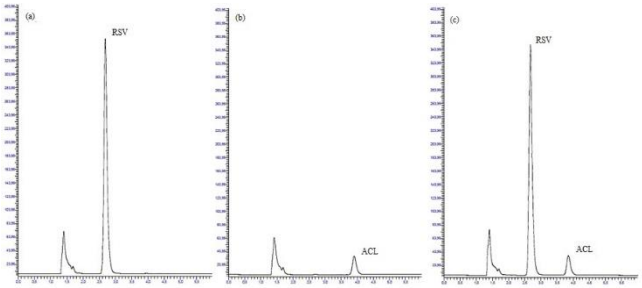

Figure 3. Chromatograms obtained for the quantification of (a) isolated RSV 25µg/mL;(b) isolate LA 75µg/mL; and (c) RSV 25µg/mL and LA 75µg/mL in association.

Specificity

The Figure 4 demonstrate the analytical method's ability to measure RSV and LA response in the presence of widely used pharmaceutical excipients and in the presence of an unloaded lipid-core nanocapsules. Thus, the method is able to quantify the aimed substances, being applicable to distinguish them in the presence of different complex matrices (22).

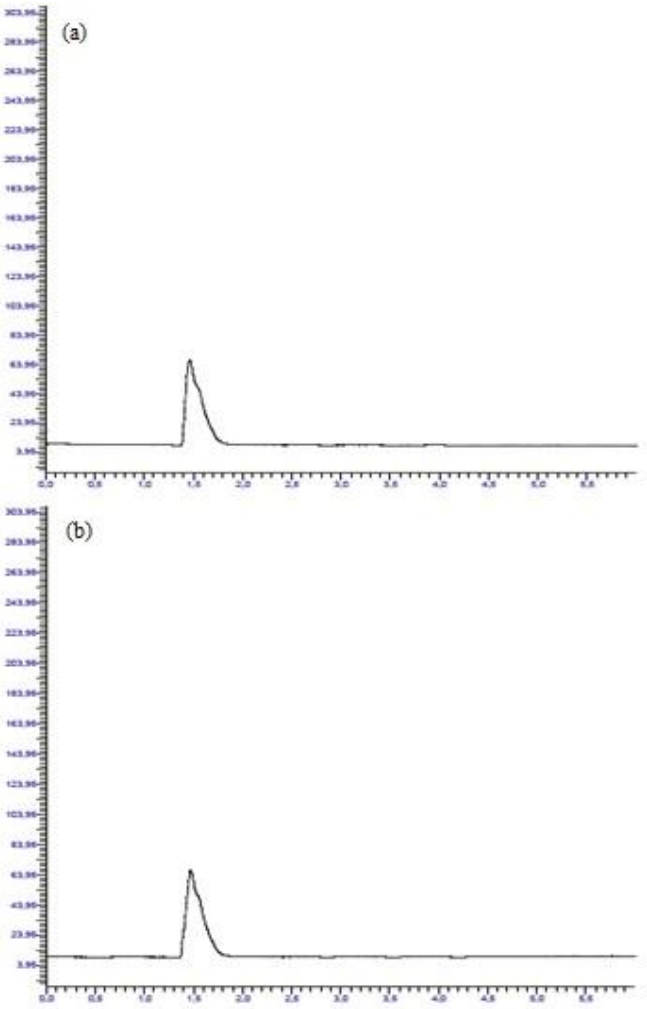

Figure 4. Chromatogram obtained from the specificity test for (a) unloaded lipid core nanocapsules and (b) excipients.

Linearity

Linearity was evidenced by the linear equations for RSV and LA (Table 2). Besides, ANOVA was calculated to verify the adjustment of the linear method and its results showed significant linear regression for both RSV and LA considering a p=0.05. In this way, the linearity results demonstrated that the results obtained experimentally are directly proportional to the concentration of the aimed substances in the sample within a specified range (22).

Table2: Validation parameters of the analytical curve obtained from HPLC method for quantitation of lipoic acid and resveratrol.

Samples	Equation	R	Value of F
LA	y = 89907x – 15119	0.996	$F_{calculated}$= 3973.9 >$F_{tabulated}$ = 4.67
RSV	y = 3372x + 2041	0.997	$F_{calculated}$= 1554.9 >$F_{tabulated}$= 4.67

Detection and quantification limits

Even though LA presents higher detection/quantification limit values than RSV, it was possible to develop a method for simultaneous quantification for both substances. The calculated detection limits for both substances demonstrated a great sensitivity for the drug detection, as shown in Table 3. In the same way, the quantification limits allowed quantification of low drug concentration, indicating that the method is sensitive enough for both drugs and, mainly, showing that in the association there was no loss in the detection and quantification limits.

Table 3: Quantification (LOQ) and Detection (LOD) limits for LA and RSV isolated or in association.

Samples	QL (µg/mL)	DL (µg/mL)
LA	6.05	2.00
LA_{ass}	6.68	2.21
RSV	1.68	0.55
RSV_{ass}	1.79	0.59

Precision

The method showed a suitable repeatability (intra-day precision) for LA and RSV, both isolated and in association, in the same day, using the same experimental conditions (Table 4). The presence of both drugs compared with the isolated drugs did not affect the repeatability, having a *p* value of 0.0566 for LA and 0.1062 for RSV.

Similarly, the inter-day precision was also adequate. The method variability is on the acceptable range (≤ 5%), indicating that the analytical method has an acceptable intermediate precision. This finding reflects the ability of the method to withstand variations in results when analysed over three different days (ICH, 1996). The experimental results are expressed as relative standard deviation (RSD) and are shown in Table 4. The presence of both drugs compared with the isolated drugs did not change the intermediate precision, having a p value of 0.0614 for LA and 0.0907 for RSV.

Table 4. HPLC-UV method's intra-day and inter-day precision for RSV and LA isolated or in association.

		Intra-day		Inter-day	
Samples	**Theoretical Concentration (µg/ml)**	**Experimental Concentration (µg/mL) ± S.D.**	**R.S.D (%)[a]**	**Experimental Concentration (µg/mL) ± S.D.**	**R.S.D (%)[b]**
LA	75	76.73±1.14	1.49	76.73±1.14	1.49
LA_{ass}	75	73.20±0.62	0.86	73.20±0.62	0.86
RSV	25	25.64±0.31	1.22	25.64±0.31	1.22
RSV_{ass}	25	25.69±0.47	1.80	25.69±0.47	1.80

[a] Average of 6 samples
[b] Average of 9 samples

Accuracy

The samples used for accuracy assay are equivalent to the lowest, intermediate and highest concentrations for LA and RSV. The results (Table 5) show the method accuracy, where the concomitant presence of the drugs did not change the drug recovery, having a p value of 0.4014 for LA and 0.5587 for RSV.

Table 5. Accuracy test by the recovery assay for RSV and LA isolated or in association, during validation of HPLC method.

Samples	Theoretical Concentration (µg/mL)	Experimental Concentration (µg/mL) ± S.D.	Average (%) ± S.D.[a]	R.S.D (%)
LA	30	29.12±1.32	98.3±1.20	1.22
	50	51.85±1.45	101.1±0.71	0.70
	120	121.38±2.01	101.1±0.81	0.80
LA_{ass}	30	29.39±1.46	98.6±1.27	1.29
	50	51.93±1.51	101.4±2.10	2.07
	120	119.21±2.23	99.3±1.97	1.98
RSV	5	5.05±0.69	100.8±2.76	2.74
	25	26.09±0.45	101.7±3.05	3.00
	50	50.23±0.98	101.3±2.59	2.55
RSV_{ass}	5	6.43±1.25	101.3±2.05	2.02
	25	25.89±1.22	100.6±1.63	1.62
	50	49.57±1.58	99.6±1.14	1.14

[a] Average of three samples

Robustness

The quantification results for RSV and LA and the experimental range of the selected variables are shown in Table 6. No significant RSD alterations were observed in the simultaneous determination of the drugs under variations of supplier, modification of MP components proportion and flow, demonstrating the robustness of the method. The robustness was evidenced for isolated substances and for the association, being observed RSD values lower than 5% for all samples.

Table 6: Method robustness, comparing different solvent manufacturers (ACN1 – Merck and ACN2- Panreac), flow and MP concentration[a] for RSV and LA isolated or in association**.**

	ACN 1		**ACN 2**	
	Average (%) ± S.D.	R.S.D.	Average (%) ± S.D.	R.S.D.
LA	100.1±0.62	0.62	99.9±0.95	0.95
LA_{ass}	99.6±0.84	0.85	98.7±0.34	0.34
RSV	98.6±1.40	1.42	99.3±0.94	0.94
RSV_{ass}	97.8±0.76	0.78	97.9±0.33	0.33
	Flow 0.7mL/min		**Flow 0.9mL/min**	
	Average (%) ± S.D.	R.S.D.	Average (%) ± S.D.	R.S.D.
LA	95.1±0.54	0.56	95.2±0.46	0.58
LA_{ass}	100.3±1.49	1.48	98.6±0.71	0.72
RSV	103.4±1.79	1.73	102.8±1.60	1.55
RSV_{ass}	104.6±0.77	0.74	102.2±1.37	1.34
	MP(65:35)		**MP(55:45)**	
	Average (%) ± S.D.	R.S.D.	Average (%) ± S.D.	R.S.D.
LA	100.6±2.60	2.58	98.6±1.10	1.12
LA_{ass}	98.7±2.12	2.15	103.3±0.91	0.88
RSV	103.2±0.38	0.37	100.2±0.15	0.15
RSV_{ass}	100.2±0.37	1.22	100.8±0.28	0.28

[a] Nominal condition was ACN/phosphoric acid (60:40) at a flow rate of 0.8 mL/min.

Conclusions

The proposed analytical method for simultaneous determination of RSV and LA was developed under official parameters (21, 22) with suitable results of specificity, linearity, precision, accuracy and robustness. The method presented a suitable detection and quantification limit for both substances. It was not observed interferences from pharmaceutical excipients as starch, magnesium stearate, lactose and mannitol and also lipid-core nanocapsules. There was no difference in the evaluated parameters when the LA and RSV were analysed isolated or in association. The developed method can be applied for the analysis of pharmaceutical dosage forms containing these substances.

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Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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