EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 5
Issue: 5
312-318
Year: 2018

RP-HPLC AND SPECTROPHOTOMETRIC METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF RESVERATROL AND L-ARGININE IN PHARMACEUTICAL DOSAGE FORM

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Article Received on 17/03/2018 **Article Revised on 07/04/2018** **Article Accepted on 28/04/2018**

ABSTRACT

A simple, rapid, economical, precise and accurate RP-HPLC method for simultaneous estimation of Resveratrol and L-Arginine has been developed. A reverse phase high performance liquid chromatoghrapic method was developes for simultaneous estimation of Resveratrol and L-Arginine. The separation was achived by LC-2010 CHT C_{18} (250 cm * 0.46 cm) Shiseido column and Buffer(pH 6.0):Methanol (40:60)as mobie phase , at flow rate of 1 ml/min. Detection was carried out at 230 nm. Retention time of L-Arginine and Resveratrol were found to be 3.143 min and 5.907 min, respectively. The method has been validated for linearity, accuracy and precision. Linearity observed for L-Arginine 5-15 µg/ml and for Resveratrol 6.25-18.75µg/ml. The percentage recoveries obtained for L-Arginine and Resveratrol were found to be in range of 100.181%-99.843% and 99.020%-98.858% respectively. Developed method was found to be accurate, precise and rapid for simultaneous estimation of L-Arginine and Resveratrol. The proposed method was successfully applied for the simultaneous estimation of both the drugs in commercial Combined dosage form.

KEYWORDS: L-Arginine, Resveratrol, Simultaneous estimation, RP-HPLC Method, Validation.

INTRODUCTION

L-Arginine (2s)-2-amino-5-(diaminomethyli-dene amino)Pentanoic acid is an amino acid.[1] L-arginine is converted in the body into a chemical called nitric oxide. Nitric oxide cause blood vessels to open wider for improved blood flow. L-arginine also stimulates the release of growth hormone, insulin, and other substance in the body and In addition L-Arginine is an Amine acid which help dispose of ammonia, is used to make nitric oxide, creatine, L-glutamate, and it converted to glucose and glycogen.[18]

Resveratrol is a antioxidants and anti inflammatory agent. The chemical name of the drug is 5-[(E)-2-(4-hydroxyphenyl)ethenyl]benzene-1,3-diol.[12] Resveratrol has antioxidant and potential chemopreventive avtivites. Resveratrol induced phase II drug-metabolizing enzyme(anti-intiation activity); mediates anti-inflammatory effect and inhibit cyclooxygenase and hydroperoxidase function.

This Combination of two drugs (esverol plus Capsule) is highly effective and used in the treatment of blood pressure and also as health supplements.

Literature survey reveals that some analytical methods have been used for the estimation of Resveratrol and L-Arginine individually or combination with other drugs. The Indian Pharmacopoeia (IP) stated the titration method for the assay of L-Arginine.[11] Resveratrol has been determined alone or with other compound in pharmaceutical formulations using High-Performance Liquid Chromatography and spectrophotometry method.[12-15] Ion chromatography[16] methods was reported for the estimation of L-Arginine alone or in combination with other drugs. Since, there is no HPLC method has been reported till date for the simultaneous estimation of L-ARG and RES in bulk and combined pharmaceutical dosage forms. Therefore the present research work, our aim is to develop a new analytical RP-HPLC method and validate according to the ICH guidelines[2] to estimate L-ARG and RES containing bulk drugs and combined pharmaceutical dosage forms in routine analysis.

MATERIALS AND METHODS

Instruments and Reagents

A double beam UV-visible Spectrophotometer (Lab India, UV-3000+), attached to a computer software UV Win, with a spectral width of 2 nm, wavelength accuracy of 0.5 nm and pair of 1 cm matched quartz cells. The

chromatography was performed by a HPLC instrument (Shimadzu LC 2010 CHT) equipped with a UV-Visible detector, injector with Isocratic pump, Sheisdo C18 column (250* 4.6mm, 5µm) and LC-solution software used. Analytical Balance and Ultra sonic cleaner were also used .The pharmaceutical preparations of combination of L-Arginine and Resveratrol that is Esverol plus Capsule (Delvin pharma Ltd.) contains 20 mg of L-Arginine and 25mg of Resveratrol was procured from local market. HPLC grade methanol (Merck Ltd., Mumbai, India), HPLC grade Acetonitrile (Merck Ltd., Mumbai, India), Analytical grade Orthophosphoric acid and water were used.

RP-HPLC Method

Chromatographic condition

Method was developed using a Sheisdo C18 column (250 mm x 4.6 mm, 5µm). Mobile phase used was potassium dihydrogen phosphate buffer (0.05 M, pH 6.0 adjusted with 0.5% orthophosphoric acid): Methanol (40:60 v/v) at flow rate is 1.0 mL/min. Samples were injected using Rheodyne injector with 20µl loop. Detection: At 230nm with UV detector.

PREPARATON OF STANDARD STOCK SOLUTION

An accurately weighed quantity of L-ARG 10mg and RES 12.5mg (Working standard drugs) were transferred to a 50ml volumetric flask and dissolved in mobile phase and finally the volume was adjusted up to the mark with mobile phase. From this stock solution working standard solution having concentration 10µg/ml and 12.5µg/ml were prepared by appropriate dilution with mobile phase for L-ARG and RES respectively.

PREPARATION OF SAMPLE SOLUTION

Twenty capsules were weighed and powder was collected. The capsules powder equivalent to 10mg of L-ARG and 12.5 mg of RES was transferred to a 50 ml volumetric flask and dissolved in mobile phase and the content was kept in ultrasonicator for 15 min. The flask was allowed to stand for 5 min at room temperature and the volume was adjusted up to the mark with mobile phase. The solution was filtered through a nylon 0.45 µm membrane filter paper. The solution was suitably diluted with mobile phase to get a final concentration of 10µg/ml of L-ARG and 12.5µg/ml RES respectively.

UV Spectrophotometry

i) Simultaneous equation method

Weighed capsule powder equivalent to 20 mg of L-Arginine and 25 mg of Resveratrol was transferred to 100 ml volumetric flask. Methanol was added, ultra-sonicated for 15min. and volume made up to mark with methanol. The solution was filtered through Whattman filter paper No.41. The filtrate was further diluted with methanol to obtain concentration 10 µg/ml of L-Arginine and 12.5 µg/ml of Resveratrol. The concentration of both L-Arginine and Resveratrol were determined by measuring the absorbances of sample at both wavelengths 238 nm and 256 nm. The concentrations of L-Arginine and Resveratrol were calculated by solving these simultaneous equations.

$$Cx = 2\ ay1 - A1\ ay2ax2\ ay1 - ax1\ ay2 \text{--------}(1)$$

$$Cy = A1\ ax2 - A2\ ax1\ ax2\ ay1 - ax1\ ay2 \text{--------}(2)$$

Where,
ax1 = Absorbance of L-Arginine at 238 nm
ax2 = Absorbance of L-Arginine at 256 nm
ay1 = Absorbance of Resveratrol at 256 nm
ay2 = Absorbance of Resveratrol at 238 nm

METHOD VALIDATION

The developed RP-HPLC and UV method was validated as per ICH guidelines.[16]

ASSAY: Twenty capsules were weighed and powder was collected. The capsule powder equivalent to 20 mg of L-ARG and 25 mg of RES was transferred to 100 ml volumetric flask. The content was mixed with mobile phase, sonicated for 15 min. Dissolve the drug completely with mobile phase. The solution was filtered through whatman filter paper no. 42 and first few drops of filtrate were discarded. The original stock solution was further diluted to get sample solution of drug concentration of L-ARG (10µg/ml) and RES (12.5µg/ml). A 20 µl volume of sample solution was injected into HPLC. The peak area for the drugs was measured at 230 nm and amount of L-ARG and RES were determined using the related linear regression equations. The % assay of the drugs was calculated and the results are given in **Table-1**.

SPECIFICITY

The specificity of the RP-HPLC method was determined by comparison of the chromatogram of mixed standards and sample solutions. The parameters like retention time (R*t*), resolution (*RS*), and asymmetry factor (*As*) and number of theoretical plates were calculated. Good correlation was found between the results of mixed standards and sample solutions.

ACCURACY

The accuracy of the method was determined by calculating the recovery studies at three levels (80%, 100% and 120%) by standard addition method. Known amounts of standard L-ARG and RES were added to the pre quantified samples and they were subjected to proposed HPLC method. The results of the recovery studies are given in **Table-4**.

PRECISION

Precision study was performed to find out intra-day and inter-day variations. In this process the combined solution (10µg/ml and 12.5µg/ml of L-ARG and RES respectively) analyzed by same day (Intra-day precision) and on three different days (Inter-day precision). The % relative standard deviation (RSD) for intra-day precision

was 0.253-0.319 % of L-ARG, 0.771-0.289 % of RES and for inter-day precision was 1.061-0.628 % of L-ARG and 1.407-1.416 % of RES respectively, which is less than 2% indicating high degree of precision.

LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ)

The LOD and LOQ for L-ARG and RES were separately determined by based on calculating the signal-to-noise ratio (S/N is 3.3 for LOD and 10 for LOQ) and from the calibration curves the standard deviation of the y-intercepts and slope of the regression lines were used. Results of LOD and LOQ are given in Table 2.

The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were derived by using the following equations designated by International Conference on Harmonization (ICH) guidelines (32).

$$LOD = 3.3 \times \sigma/S$$

$$LOQ = 10 \times \sigma/S$$

Where, σ = the standard deviation of the response
S = slope of the calibration curve.

LINEARITY

An accurately weighed quantity of L-ARG 100mg and RES 125mg (Working standard drugs) were transferred to into a separate 50mL clean and dry volumetric flasks and dissolved in mobile phase and finally each volumetric flask volume was adjusted up to the mark with mobile phase respectively. From this stock solution prepare 5, 7.5, 10, 12.5 and 15µg/ml of L-ARG and 6.25, 9.37, 12.5, 15.62 and 18.75µg/ml of RES concentrations respectively. Inject each level into the chromatographic system and measure the peak area. Plot a graph of peak area versus concentration (on X-axis concentration and on Y-axis Peak area) and calculate the correlation coefficient. The results were shown in **Table-5**.

ROBUSTNESS

The robustness study was done by making small changes in the optimized method parameters like changing in pH of the mobile phase by ± 2%, mobile phase ratio by ±2%, and flow rate by ± 2 ml/min and the chromatographic characteristics were evaluated. No significance change was observed.

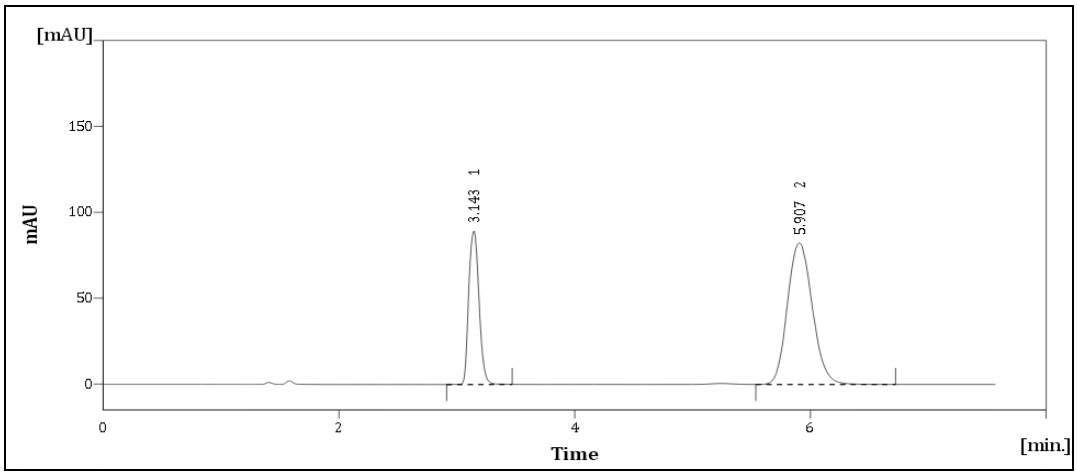

Figure 1: Chromatogram of L-ARG and RES of std. Solution.

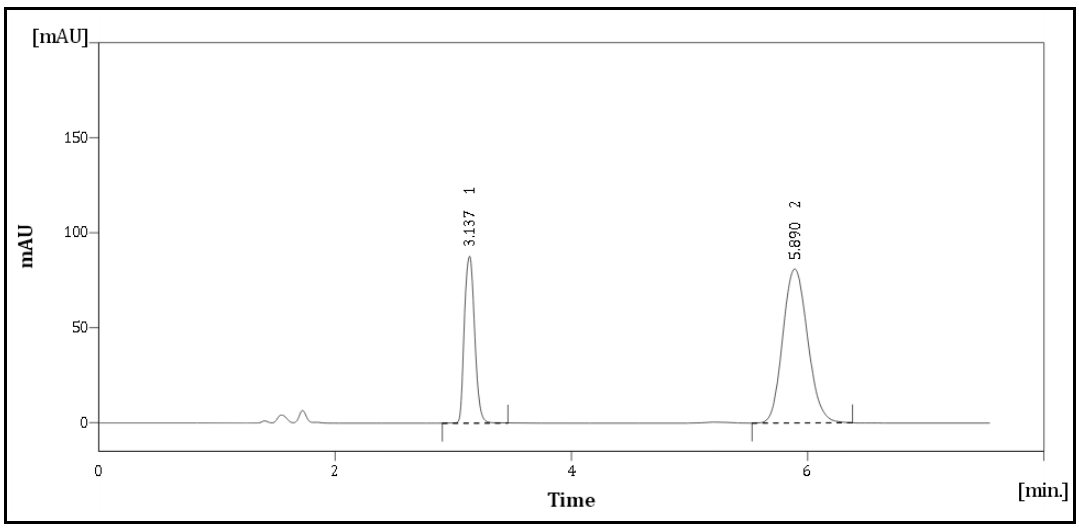

Figure 2: Chromatogram of L-ARG and RES of sample solution.

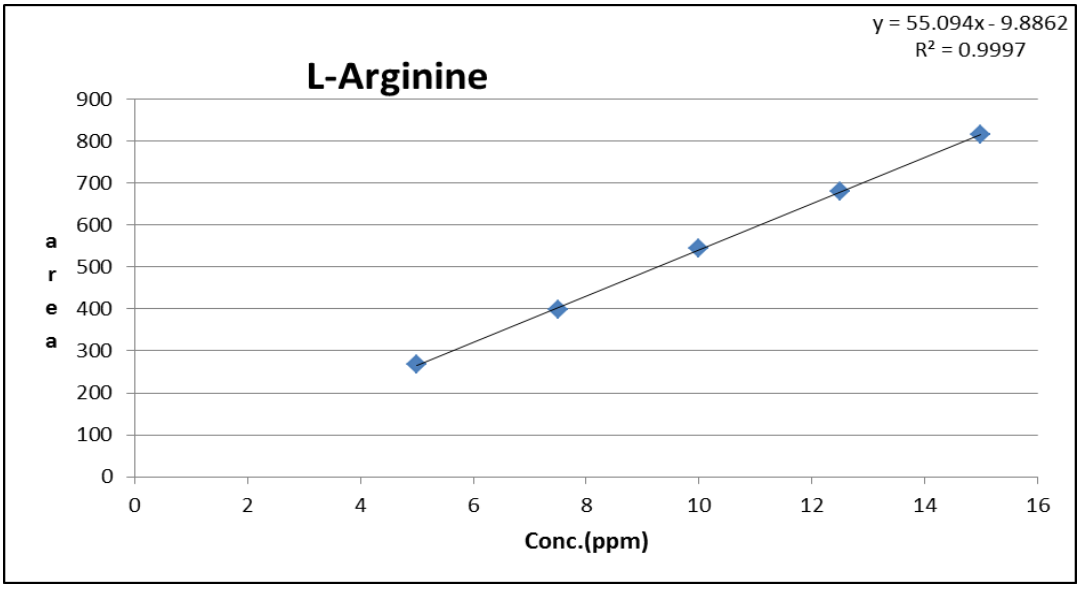

Figure 3: Calibration curve of L-Arginine.

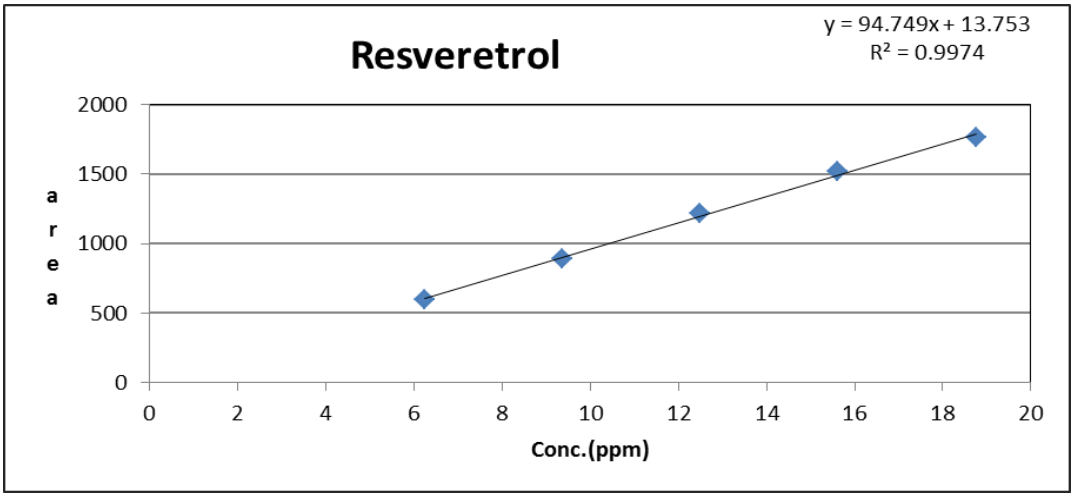

Figure 4: Calibration curve of Resveratrol.

FOR UV SPECTROPHOTOMETRIC

- **Simultaneous equation method**

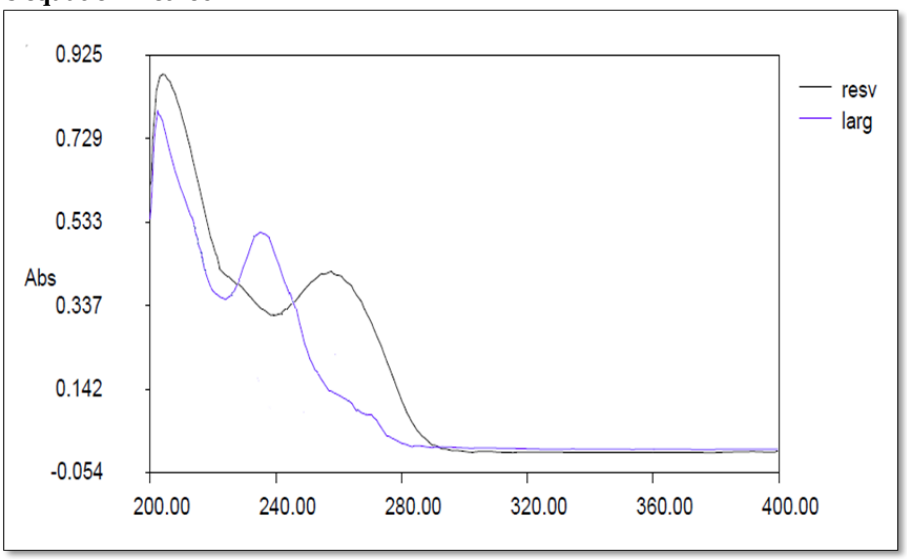

Figure 5: Overlain spectra of L-Arginine and Resveratrol.

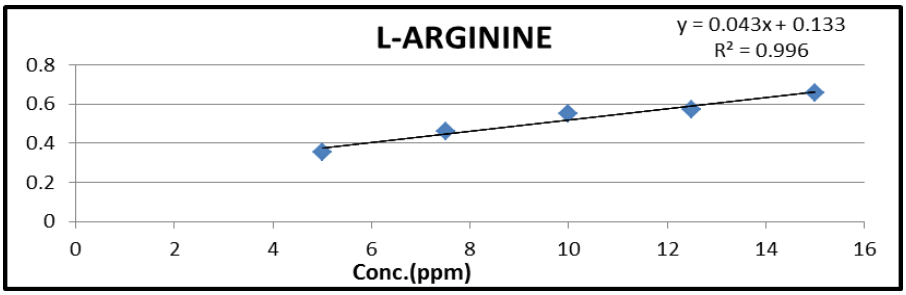

Figure 6: Calibration curve of L-Arginine at 238 nm.

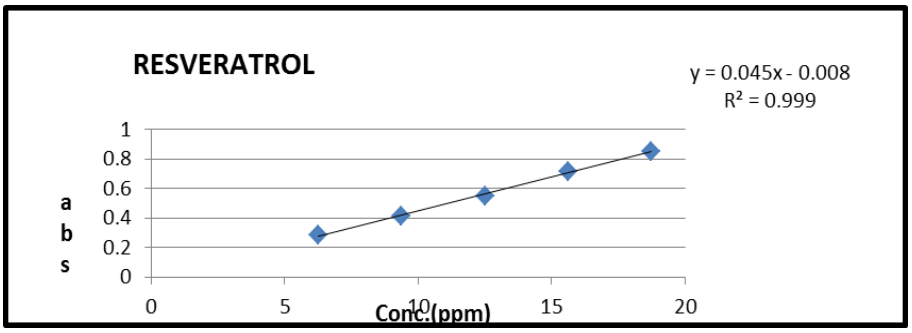

Figure 7: Calibration curve of Resveratrol at 256 nm.

Table 1: Assay Parameters.

Formulation (capsule)	Capsule amount (mg)		Amount found (mg)		% Assay	
	L-ARG	RES	L-ARG	RES	L-ARG± S.D (n=3)	RES±S.D (n=3)
1	20	25	19.64	24.44	97.928 ±0.317	97.802± 0.299
2	20	25	19.62	24.52		
3	20	25	19.52	24.37		

Table 2: Results from validation and system suitability studies.

Validation parameters	L-ARG	RES
Theoretical plates	5457	3550
Resolution	-	9.756
Asymmetry factor	1.273	1.193
Intra-day precision (%RSD)	0.253-0.319	0.771-0.289
Inter-day precision (%RSD)	1.061-0.628	10407-1.416
LOD (µg/ml)	0.246	0.953
LOQ (µg/ml)	0.745	2.290

Table 3: Result from validation and system suitability studies of simultaneous method.

Validation parameters		L-ARG	RES
Wavelength for measurement		238 nm	256 nm
Beer's law limit		5-15 µg/ml	6.25-18.75 µg/ml
Regression equation		Y = 0.043x + 0.133	Y=0.045X- 0.008
Correlation coefficient		0.996	0.999
% Recovery	80%	98.410-97.490	98.790-101.478
	100%	99.755-100.733	97.845-96.774
	120%	98.410-98.207	100.357-99.462
LOD(µg/ml)		1.346	0.560
LOQ(µg/ml)		4.080	1.698
Repeatability (% RSD, n=6)		0.735	0.360
Interday Precision (%RSD)(n=3) at 3 diff. conc.		0.536-0.310	0.535-0.234
Intraday Precision(%RSD)(n=3)		0.462-0.233	0.733-0.565

Table 4: Accuracy.

Drug	Amount of sample taken(µg/ml)	Amount of standard spiked (µg/ml)	Total amount (µg/ml)	Amount recovery (mg)	% recovery (mg)
L-ARG	5	4	9	4.02	100.695
	5	5	10	4.99	99.848
	5	6	11	5.98	99.721
RES	6.25	5	11.25	4.98	99.763
	6.25	6.25	12.5	6.23	99.760
	6.25	7.5	13.75	7.47	98.628

Table 5: Linearity.

SR.NO	Concentration(µg/ml)		Area	
	L-ARG	RES	L-ARG	RES
1	5	6.25	267.002	598.571
2	7.5	9.37	398.029	887.866
3	10	12.5	544.968	1219.241
4	12.5	15.625	679.596	1521.326
5	15	18.75	815.457	1762.362

SUMMARY AND DISCUSSION

To optimize the RP-HPLC parameters, several mobile phase compositions were tried. A satisfactory separation and good peak symmetry for L-ARG and RES was obtained using Kromasil Stainless steel C18 G column (250 mm ×4.6 mm, 5µm) with a mobile phase consisting of potassium dihydrogen phosphate buffer (0.05 M, pH 6.0 adjusted with 0.5% orthophosphoric acid): methanol (40:60 v/v) at flow rate is 1.0 mL/min, PDA detection was performed at 230nm. The retention times of L-Arginine and Resveratrol were found to be 3.137 min and 5.890 min respectively (**Figure 1**). The amount of L-ARG and RES present in the sample solutions were determined respectively and the results obtained were comparable with the corresponding labeled claim (**Table 1**). The results of system suitability testing are given in Table 2. The %RSD of L-ARG and RES for intra-day precision and inter-day precision was less than 2% it reveal that the proposed method is precise (**Table 2**). The sensitivity of method LOD and LOQ is shown in **Table 2**. The % recovery was found to be 98-102% within the limits for L-ARG and RES (**Table 4**) which indicates high degree of accuracy of developed method. Linear correlation was obtained between concentration versus peak area of L-ARG and RES in the concentration ranges of 5-15(µg/ml) and 6.25-18.75(µg/ml) respectively (**Table 5**). The correlation co-efficient (' *r2* ' value) for L-ARG and RES was 0.997 and 0.999 respectively. The results of the robustness study also indicated that the method is robust and is unaffected by small variations in the chromatographic conditions.

For simultaneous equation method the absorbance was found to be 238 nm and 256 nm of L-ARG and RES against Methanol(**Figure 5**). The calibration curve was found to be linear in the concentration range of 5-15µg/ml and 6.25-18.75 µg/ml for L-Arginine and Resveratrol (**Figure 6,7**), respectively. The result of validation parameters are given in Table 3.

CONCLUSION

The proposed study describes RP-HPLC and UV method for the estimation of L-ARG and RES in capsule formulation. The method was validated according to the ICH guidelines. Hence, it can be concluded that the developed RP-HPLC and UV method is accurate, precise, and selective and it can be employed successfully for the estimation of L-ARG and RES in the capsule formulation in routine analysis.

ACKNOWLEDGEMENT

The authors are grateful to Department of Qualty Assurance, smt B.N.B Swaminarayan Pharmacy College, Salvav, Vapi, affliated to Gujarat technological university, Ahmadabad, India for providing all the facilities to carry out the work.

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