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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR SIMULATANEOUS ESTIMATION OF RESVERATROL AND ORLISTAT IN BULK AND SYNTHETIC MIXTURE

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ABSTRACT

A simple, precise, rapid and accurate RP-HPLC method was developed for the estimation of Resveratrol and Orlistat. ZORBAX Eclipse plus C_{18} Column (100mm x 4.6 mm, 3.5µm) with mobile phase consisting of mixture of acetonitrile: water (90:10) and pH adjusted to 5 with Ortho-phosphoric acid. The flow rate was 0.8 ml/min and the effluents were monitored at 305 nm for RES, 210 nm for ORL. The retention time was 10 min. The detector response was linear in the concentration of 5-30 and 20-80 µg/ml for RES and ORL respectively. The respective linear regression equation being y=88761x+139736 and y=39240x+366015 for RES and ORL respectively .The limit of detection (LOD) for RES and ORL were found to be 0.86 and 0.37. While limit of quantification (LOQ) for RES and ORL were found to be 2.86 and 1.23 respectively. The percentage assay of RES and ORL was 100%. The repeatability, intraday and inter day were found in the limit as per ICH guidelines. Thus developed method was precise. The method can be successfully employed for the simultaneous determination of RES and ORL in fixed dose pharmaceutical formulations. ICH guidelines Q2(R1) is considered for the validation of RP-HPLC method.

Keywords: Resveratrol, Orlistat, RP-HPLC method development and ICH guidelines.

INTRODUCTION

The number of drugs and drug formulations introduced into the market has been increasing at an alarming rate. These drugs or formulations may be either new entities or partial structural modification of the existing ones or novel dosage forms. The US FDA US9433602patent is approved for fixed dosage combination of RES (Resveratrol) and ORL (Orlistat) as antidiabetic herbal formulation.[1-3] One of the uses for the combination therapy is antidiabetic herbal formulation. The reassessment of literature supports that a range of HPLC, HPTLC and UV-visible Spectrophotometric methods have been described for quantification of RES and ORL separately or in blend with other drugs/phytomarkers. It is also made known that 'no Spectroscopic method, HPLC or HPTLC method has been accounted in the literature for estimation of RES and ORL combination'. The condition thus, imparted the prospect of development of reproducible analytical methods capable of estimating both RES and ORL simultaneously. [4-13]

EXPERIMENTAL

Apparatus and Instrument

Digital weighing balance– Denver SI234, Germany

UV Spectrophotometer: Shimadzu – UV 1800, Japan

HPLC System

- Model: Agilent 1260 series HPLC
- Make: Agilent Technologies, USA

- Injector: Rheodyne, 6 port manual injector
- Sample loop: 20 µl fixed volume loop
- Pump: Quat pump VL
- Detector: Diode array detector

CHEMICALS AND REAGENTS

- Resveratrol reference standard (Purchased from Sigma Aldrich and gift sample provided by Zydus Cadila, Ahmedabad)
- Orlistat reference standard (Gift sample provided by Bills biotech Pvt. Ltd., Vadodara)
- All other solvents taken were from the Merck India Limited.

Preparation of Stock Solutions

To Prepare stock solution of RES (500 µg/ml) and ORL (1000 µg/ml), accurately weigh 50mg for RES and 10 mg of ORL were transferred in two different 100 and 10 ml previously calibrated volumetric flasks, dissolve and 5 ml methanol was added to the volumetric flask, it was than diluted up to the mark with methanol. from these stock solutions, 2 ml and 2.5 ml aliquots of RES and ORL respectively were transferred in two different 10 ml previously calibrated volumetric flasks and were diluted up to mark with methanol to get working standard solution having concentration of RES of 100 µg/ml and ORL of 250 µg/ml.

Calibration Curve for RES and ORL

Linearity of the method was checked using working standard solution having concentration in range 0.5, 1, 1.5, 2, 2.5, 3 ml from RES working standard and 0.8, 1.4, 2, 2.6, 3.2, 3.8 ml from ORL working standard were transferred in different volumetric flask of 10ml and diluted with methanol up to the mark to obtain final concentration range of 5-30 µg/ml for RES and 20-95 µg/ml for ORL.

Preparation of RES and ORL working standard mix solution

2 ml from RES working standard solution and 5 ml from ORL working standard solution were transferred to 10 ml of previously calibrated volumetric flask. Then it was diluted up to the mark with methanol to get final working standard mix solution of 20 µg/ml RES and 50 µg/ml ORL.

Preparation of sample solution from laboratory prepared synthetic mixture:

Synthetic mixture was prepared containing 500 mg RES, 120 mg ORL, 20 mg microcrystalline cellulose, 90 mg sodium starch glycolate, 60 mg povidone, 20 mg sodium lauryl sulphate and 20 mg talc per capsule. From the prepared mixture, amount equivalent to 20 mg RES was added to 100 ml volumetric flask containing 45.2 mg of ORL standard. Then it was sonicated for 15 min. and filtered through Whatman filter paper. From this solution, 5 ml was transferred in 10 ml volumetric flask and diluted with methanol up to the mark. From this solution, 2 ml was transferred in 10 ml volumetric flask and diluted up to the mark with methanol to make final concentration of RES and ORL, 20 µg/ml and 50 µg/ml respectively. It was then filtered through 0.45 µm syringe filter discarding first 1-2 ml filtrate. Respectively which was used for assay.

EXPERIMENTAL

Selection of Detection Wavelength

The standard solutions of individual RES and ORL was scanned in UV Spectrophotometer to select wavelength for the analysis. In present study, λmax of RES and ORL were selected as detection wavelength for RES and ORL which were 305 nm and 210 respectively. Figure 1 (a and b) represents UV Spectra of 20 µg/ml RES and 50 µg/ml ORL using PDA detector of HPLC respectively.

Optimization of Mobile Phase

Combination of methanol: water (60:40, v/v) was tried to elute RES and ORL which was unsuccessful as ORL did not elute out. In the next trial ACN: methanol: water (20:40:40, v/v/v) was tried, but ORL peak shape was asymmetric which was unacceptable to final the trial. In the further trial, ACN:water (60:40, v/v) was tried and it was seen that both the phytomarkers got separated but ORL peak shape was still asymmetric. In the next trial, methanol:water (pH 5 adjusted with OPA) in the ratio of (60:40, v/v) was used. In this trial, both peaks were separated but ORL eluted too late. ORL Peak symmetry was poor. In the fifth trial, ACN: water (pH 5 adjusted with OPA) was used to reduce ORL retention time as well as to achieve proper peak shape of ORL. Both peaks were well separated with proper peak shapes. To modify the retention time, in the next trial, ACN: water (pH 5 adjusted with OPA) (90:10, v/v) was used, where RES and ORL both peaks with good resolution and symmetry with optimum retention times were obtained. Chromatograms at 305 nm for RES and Chromatograms at 210 nm for ORL were shown in Figure 2 (A-F) and Figure 2 (a-f) respectively. The optimized chromatographic conditions are shown in Table 1.

Chromatographic condition

RESULTS AND DISCUSSION

Validation of the Proposed Method

1.Linearity

Linear correlation was obtained between absorbance Vs concentration of RES and ORL in the concentration ranges of 5- 30 µg/ml and 20 - 95 µg/ml respectively and is shown in overlain chromatogram of RES and ORL in figure 2. Calibration curve data of RES and ORL shown in Table 2. Regression parameters are mentioned in Table 3 and Figure 2 (A-F) (at 305 nm for RES) and Figure 2 (a-f) (at 210nm for ORL) represent standard chromatograms of each level of linearity. The areas obtained are directly proportional to the concentration of analyte of interest.

2. Accuracy

Accuracy was determined by calculating the % recovery of RES and ORL from the synthetic mixture by the standard spiking method. Percentage recovery for RES was in the range of 99.24-100.19%**,** while for ORL, it was found to be in range of 99.28-99.99 %. The results are shown in Table 4 and 5. Recovery greater than 99 % with low SD justifies the accuracy of the method.

3. Precision

3.1 Repeatability

Each concentration level of linearity range for RES (5-30 µg/ml) and ORL (20-95 µg/ml) were injected without changing the parameters. This same procedure was replicated five times. The %RSD values obtained were ranging from 0.14% to 0.48% for RES and 0.12% to 0.66% for ORL. Table 6 and Table 7 represent method precision data for RES and ORL respectively.Table Represent % R.S.D was less than 2% complied with the standard limits.

3.2 Intra-day and inter day precision

Three different concentrations of standards RES (15, 20, 25 µg/ml) and ORL (50, 65, 80 µg/ml) were analyzed in triplicate manner at different times on the same day for intra-day and at different times on same days over a period of one week for inter-day precision. %RSD of mean areas for both the phytomarkers were calculated to check the precision. The %RSD values calculated from mean areas and S.D. were 0.33-0.58% for RES and 0.23-0.64% for ORL. Table 8 represents the data of both RES and ORL. As the %RSD values are less than 2%, the proposed method is adequately precise.

4. Sensitivity

The sensitivity of method is determined in terms of LOD and LOQ. LOD and LOQ is calculated from slop of calibration curve and S.D. of response values. The LOD for RES and ORL were found to be 0.86 µg/ml and 0.37 µg/ml respectively. The LOQ for RES and ORL were found to be 2.86 µg/ml and 1.23 µg/ml respectively. Table 9 represents the data of sensitivity.

5. Analysis of Synthetic mixture

20 µl of sample mix solution was injected in HPLC system. The procedure was replicated six times. The mean % purity for RES and ORL were found to be 99.83% and 99.80% respectively. The results of assay suggest that the method can be suitably applicable to formulation mixture too. Table 10 represents concentration obtained and % purity calculated for RES and ORL. Figure 1(a, b) represents chromatogram of RES and ORL from synthetic mixture.

CONCLUSION

The estimation of RES and ORL can be done in the presence of pharmaceutical additives using the proposed method suggest its applicability to formulations for estimation of RES and ORL simultaneously. The results of validation parameters indicate the implementation of method for the routine analysis. The results of the analysis of standards and sample mixture by proposed method were found to be substantially reproducible and accurate. Hence the proposed validated method is appropriate for routine analysis in quality control labs.

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Table 1: Optimized Chromatographic Condition

Parameter	Optimized Chromatographic Conditions
Column	ZORBAX Eclipse plus C_{18} Column, 100mm x 4.6 mm, 3.5µm
Mobile Phase	ACN: water (pH 5 adjusted with OPA) (90:10)
Flow rate	0.8 ml/min
Detection	305 nm for RES, 210 nm for ORL
Injection Volume	20 µL
Temperature	Ambient
Run time	10 min

Table 2: Result of Calibration curve data for RES & ORL

Concentration (µg/ml)	Area Mean (n=5) ± SD*	%RSD	Concentration (µg/ml)	Area Mean (n=5) ± SD*	%RSD
5	602696 ± 2924	0.49	**20**	444555 ± 3696	0.83
10	994179 ± 3735	0.38	**35**	995160 ± 5661	0.57
15	1489560 ± 11978	0.80	**50**	1584394 ± 11251	0.71
20	1889639 ± 8682	0.46	**65**	2178594 ± 18027	0.83
25	2391058 ± 11967	0.50	**80**	2391058 ± 11967	0.50
30	2791187 ± 11690	0.42	**95**	3372740 ± 2891	0.09

***=Mean± SD (n=5)**

Table 3: Statistical Data of RES and ORL

Parameters	Results	
	RES	**ORL**
Linear Range(µg/ml)	5-30	20-95
Slope	88761	39240
Intercept	139736	366015
Regression Equation	y = 88761x + 139736	y = 39240x + 366015
Co-relation co-efficient (r2)	0.9991	0.9997

Table 4: Recovery Data of RES

% Level	Concentration of RES in sample (µg/ml)*	Concentration of RES recovered (µg/ml)	% Recovery of RES (%Recovery ± SD) **	%RSD (%)
80%	16	16.00	99.92 ± 0.57	0.57
	16	15.89		
	16	16.07		
100%	20	20.07	99.98 ± 1.53	1.53
	20	20.26		
	20	19.66		
120%	24	24.11	100.19 ± 0.30	0.30
	24	23.97		
	24	24.06		

***= concentration is expressed in µg/ml, **= recovery is expressed in %Recovery ± SD**

Table 5: Recovery Data of ORL

% Level	Concentration of ORL in sample (µg/ml)	Concentration of ORL recovered (µg/ml)	% Recovery of ORL (%)	%RSD (%)
80%	40	40.01	99.28 ± 1.35	1.35
	40	40.00		
	40	39.12		
100%	50	50.07	99.99 ± 0.12	0.12
	50	50.26		
	50	49.66		
120%	60	60.01	99.64 ± 1.09	1.09
	60	59.89		
	60	59.45		

***= concentration is expressed in µg/ml, **= recovery is expressed in %Recovery ± SD**

Table 6: Method repeatability Data of RES

Conc.*	5	10	15	20	25	30
Area	601182	949992	1891656	1899543	2378922	2771100
	601184	959222	1891111	1899989	2379222	2799777
	602222	958888	1890999	1891845	2379931	2792934
	607818	957873	1898999	1899666	2372210	2799123
	605274	951222	1897991	1891000	2375210	2792999
Mean	603536	955439	1894151	1896409	2377099	2791187
S.D.	2922	4460	3989	4564	3291	11689
%RSD	**0.48**	**0.47**	**0.21**	**0.24**	**0.14**	**0.42**

***= concentration is expressed in µg/ml**

Table 7: Method repeatability Data of ORL

Conc. *	20	35	50	65	80	95
Area	441167	999002	1581786	2175643	2796722	3374522
	441184	993222	1581111	2179989	2791222	3372222
	442222	993888	1590999	2172145	2799931	3371115
	447815	993873	1588999	2179666	2779210	3378733
	445274	991222	1597991	2171000	2779210	3379933
Mean	443532	994241	1588177	2175689	2789259	3375305
S.D.	2924	2876	6995	4149	9688	3900
%RSD	**0.66**	**0.29**	**0.44**	**0.19**	**0.35**	**0.12**

***= concentration is expressed in µg/ml**

Table 8: Intra-day and inter day precision data of RES and ORL

Intra-day precision				Inter -day precision			
RES		ORL		RES		ORL	
Conc.*	%RSD	Conc. *	%RSD	Conc. *	%RSD	Conc. *	%RSD
15	0.25	**50**	0.64	**15**	0.80	**50**	0.73
20	0.17	**65**	0.23	**20**	0.52	**65**	1.38
25	0.20	**80**	0.38	**25**	0.25	**80**	0.68

***= concentration is expressed in µg/ml**

Table 9: Results of Sensitivity Data for RES and ORL

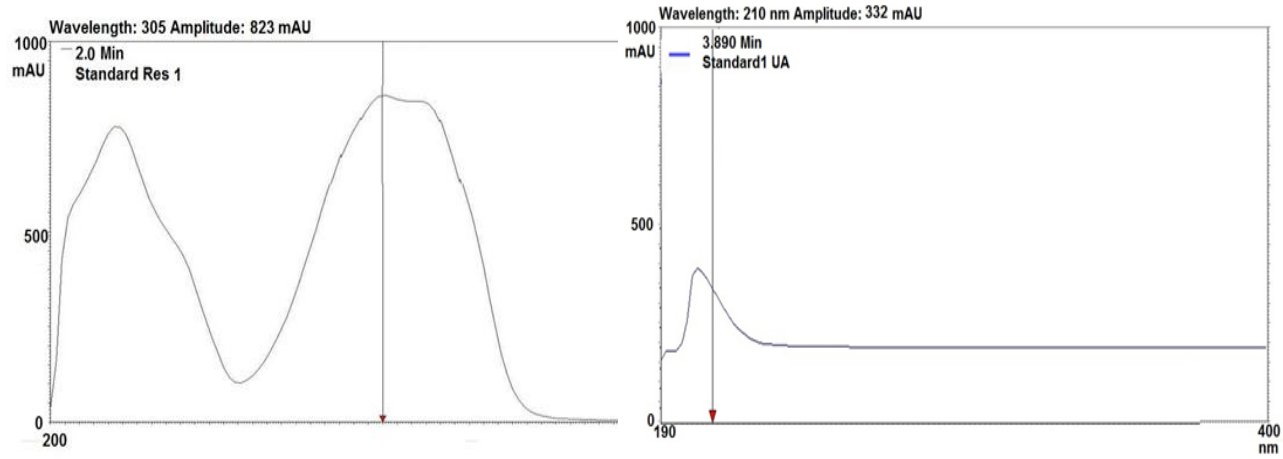
Parameter	Results	
	RES	ORL
LOD (µg/ml)*	0.86	0.37
LOQ (µg/ml)*	2.86	1.23

***= LOD and LOQ are expressed in µg/ml**

Table 10: Assay of Synthetic Mixture

Parameters	RES	ORL
Actual Concentration (µg/ml)*	20	50
Concentration Obtained (µg/ml)*	19.97	49.90
%Purity	99.83 ± 0.45	99.80 ± 0.63

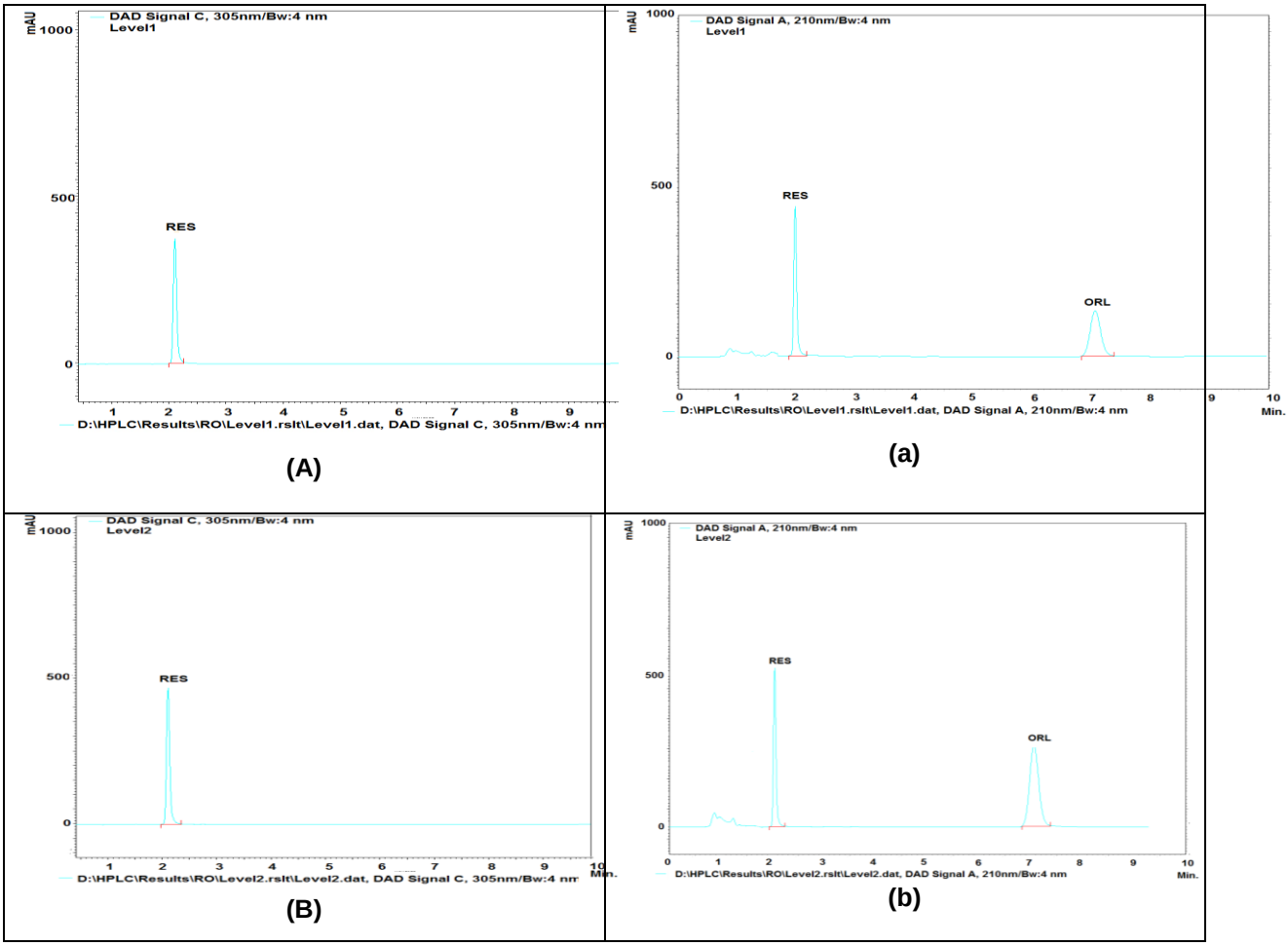
***= concentration is expressed in µg/ml**

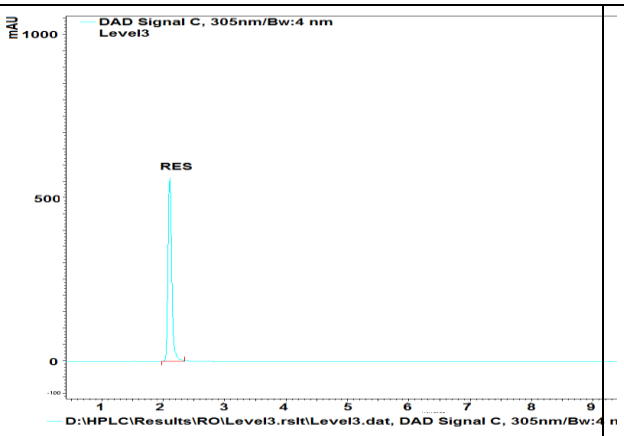


a **b**

Fig. 1(a,b): UV Spectra of RES (20 µg/ml) and ORL (50 µg/ml)

Figure 1a is UV spectra of RES and Figure 1b is UV spectra

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RES
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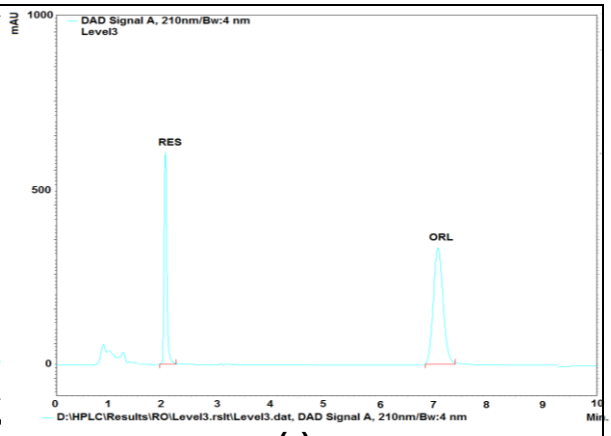
(C)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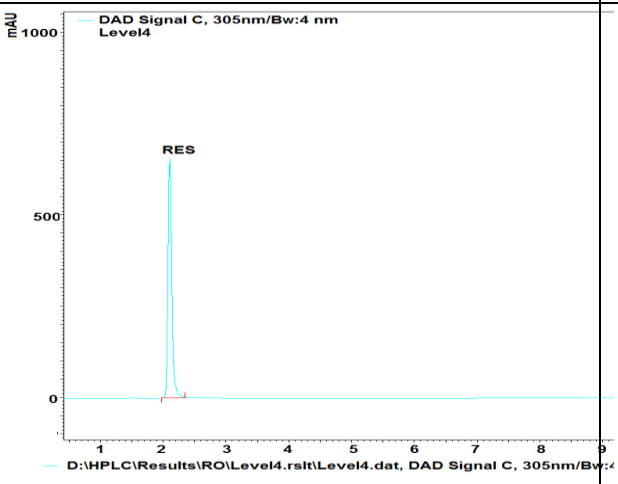
(c)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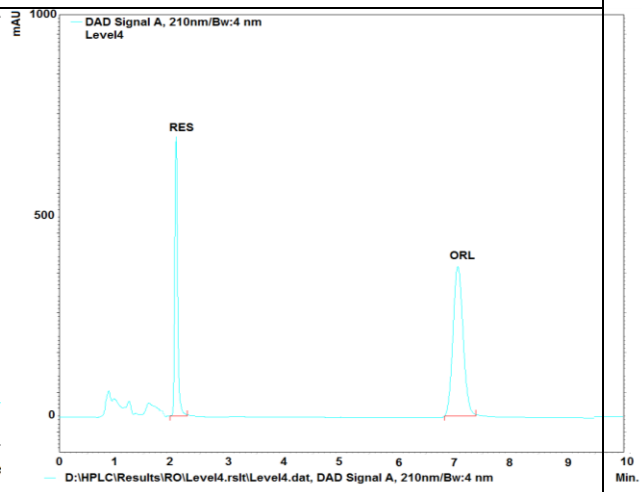
(D)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ORL
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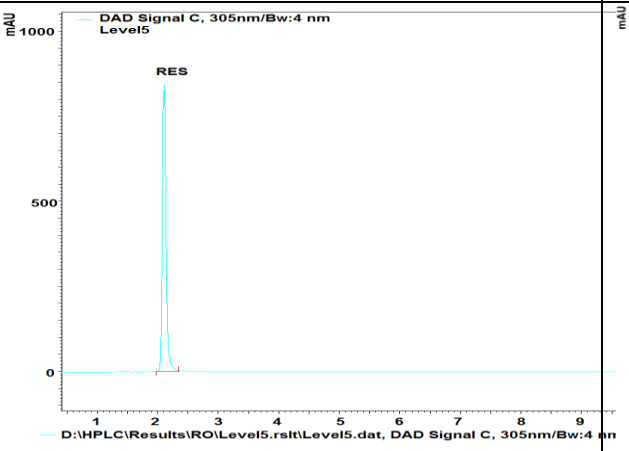
Min.

(d)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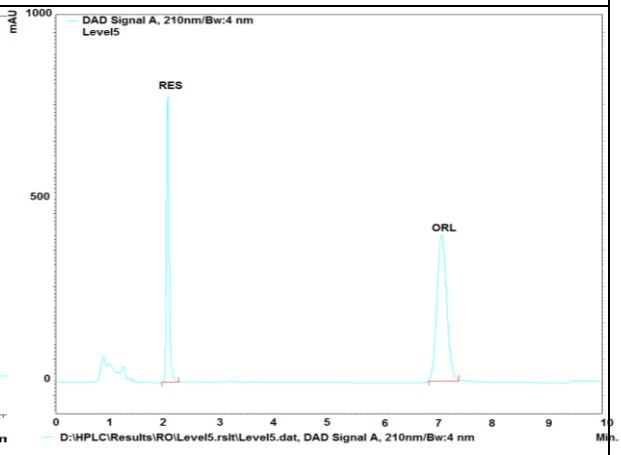
(E)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Min.

(e)

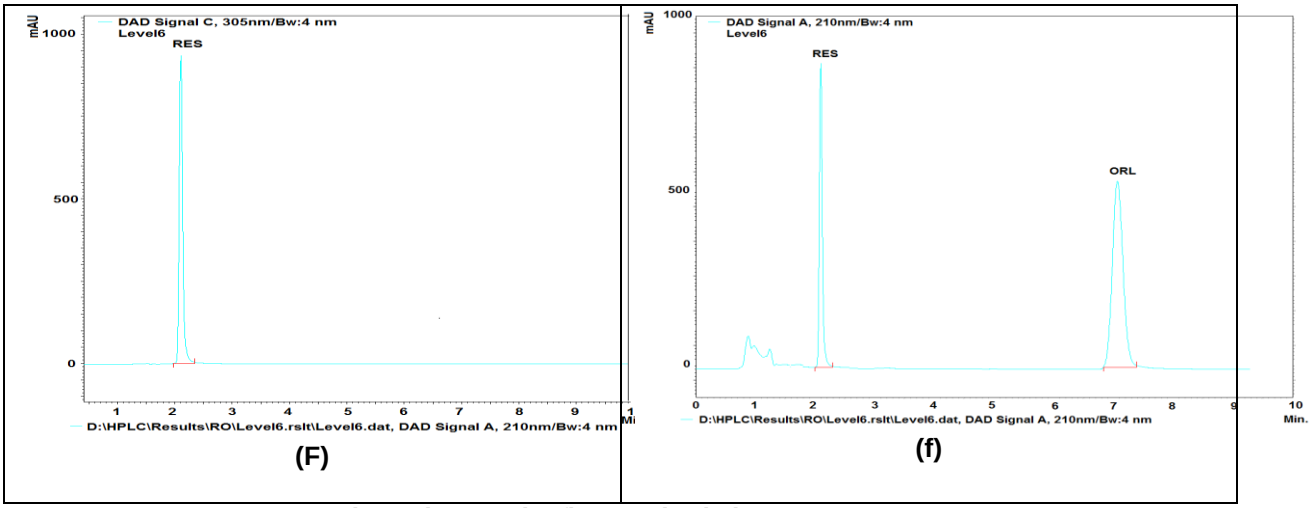
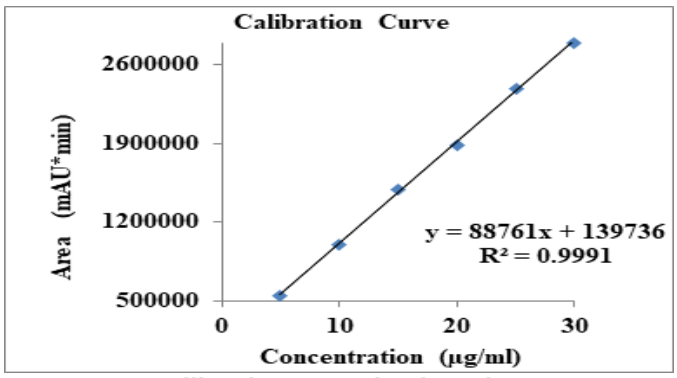
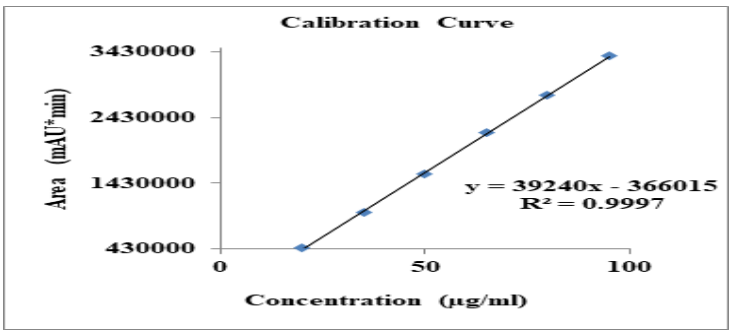


Fig. 2: (A-F and a-f): Standard chromatogram of RES (5-30 µg/ml) and ORL (20-95 µg/ml)



Calibration curve is plotted as Area(mAU*min) vs Concentration(µg/ml)
Fig. 3: Calibration Curve of RES



Calibration curve is plotted as Area(mAU*min) vs Concentration(µg/ml)
Fig. 4: Calibration Curve of ORL

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