



**DEVELOPMENT AND VALIDATION OF UV-SPECTROSCOPIC
METHOD FOR SIMULTANEOUS ESTIMATION OF L-
GLUTATHIONE REDUCED AND α - LIPOIC ACID IN TABLET
DOSAGE FORM USING DICHLONE AS A DERIVATIZING REAGENT**

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ABSTRACT

The present work aims at development of a novel method for the simultaneous estimation of L-Glutathione reduced and α - Lipoic acid using dichlone as a derivatizing reagent. As L-Glutathione reduced has various pharmacological uses like its activity against liver disorders, cancer and as an anti-aging agent. Similiarly, α -lipoic acid is of a major use in pain of liver disorders and diabetic neuropathy and it also increases the production of glutathione in the body, hence a proper analytical method for the fixed dose and its validation method is necessary for combined formulation. Dichlone forms a complex with both the drugs and its estimation is done in the visible region (400-800 nm) using Absorbance correction method.

KEYWORDS: L-Glutathione reduced, α - Lipoic acid, dichlone as a derivatizing reagent, Absorbance correction method.

L-Glutathione Reduced: The IUPAC name of L-Glutathione is (2S)-2-Amino-4-[[[(1R)-1-[(carboxymethyl)carbamoyl]2- sulfanylethyl] carbamoyl] butanoic acid. It belongs to the thiol class of drugs. It is generally abbreviated as GSH.¹ Glutathione exists in both reduced (GSH) and oxidized (GSSG) states. It is used in wine making, cosmetics (as a Skin whitening agent), Glutathione is also needed for the detoxification of methylglyoxal, a toxin produced as a by-product of metabolism.^[2]

Alpha Lipoic ACID: IUPAC name of α -lipoic acid is (R) -5-(1, 2-dithiolan-3-yl) pentanoic acid which is an antioxidant. It also has been used for cancer, liver ailment and various other conditions. People with diabetes use α - lipoic acid to treat nerve damage to the hands and feet (peripheral neuropathy). It decreases oxidative stress and lessens the symptoms such as pain. It is an 'antioxidant', a substance that protects cell from the damaging effects of oxidative stress. It is produced in the liver, it is found in foods like broccoli, spinach, potatoes, yeast and animal liver.^[3]

L-Glutathione Reduced and Alpha Lipoic ACID Formulation

The combination of L-Glutathione (reduced glutathione) and α -lipoic acid is an anti-oxidant combination that is used for beautifying the skin and protects the skin from free radicals. Also, α - lipoic acid enhances the production of L-glutathione inside the body.^[4,5]

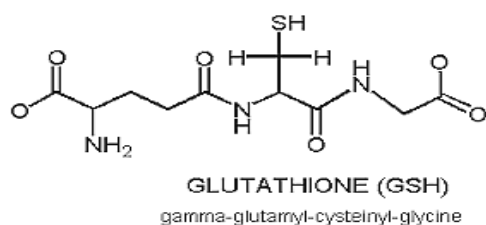


Fig. 1. L-Glutathione reduced¹.

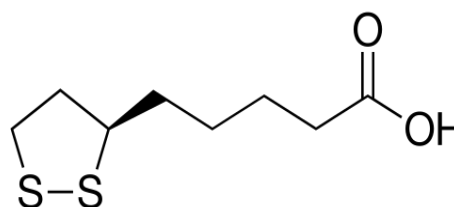


Fig. 2. α - Lipoic acid¹.

MATERIALS AND METHODS

Materials: Both the API were procured as: L-Glutathione reduced purchased from HI Media Gujarat, India and Alpha lipoic acid purchased from SRL Laboratories Gujarat, India.

Instrumentation

Instruments used in the study were

UV-visible spectrophotometer (Shimadzu 1800- Double beam spectrophotometer) with Quartz cuvette pair with 1 cm path length at 800 – 200 nm scanning range.

Experimental

1. Absorbance correction method after derivatization with dichlone reagent^[6]

Preparation of solution of Dichlone reagent

Accurately weighed 25 mg of Dichlone was transferred into 100 ml volumetric flask. To this 2ml of N,N-dimethyl formamide was added. Diluted up to the mark with ethanol. The solution was then sonicated for about 15 min.

Preparation of working standard solution of L-Glutathione reduced: Accurately weighed quantity of 50 mg of L-Glutathione reduced was transferred into 10 ml volumetric flask and diluted up to mark with ethanol to obtained final concentration 5000 µg/ml L-Glutathione reduced. Solution was sonicated for 15 minutes. Solution was further diluted with Ethanol to obtained working standard solution of 500 µg/ml L-Glutathione reduced.

Preparation of working standard solution of Alpha lipoic acid

Accurately weighed quantity of 10 mg of Alpha lipoic acid was transferred into 10 ml volumetric flask and diluted up to mark with ethanol to obtained final concentration 1000 µg/ml Alpha lipoic acid. Solution was sonicated for 15 minutes. Solution was further diluted with Ethanol to obtained working standard solution of 100 µg/ml Alpha lipoic acid.

Preparation of calibration curve for L-Glutathione reduced and Alpha lipoic acid

Standard solutions having concentration 20, 30, 40, 50 and 60 µg/ml for Alpha lipoic acid and 100, 150, 200, 250 and 300 µg/ml for L-Glutathione reduced were prepared. To this, 1 ml dichlone reagent was added and diluted upto mark with ethanol. Absorbance of prepared standard working solutions were measured at 365 nm and 430 nm. Standard calibration curves of absorbance against concentration were plotted. Absorptivity coefficients were determined using calibration curves at both the wavelengths.

METHOD VALIDATION

Validation of developed method was carried out according to ICH guideline for Validation of Analytical Procedures Q2 (R1).

Linearity: Solutions having concentration 20, 30, 40, 50 and 60 µg/ml for Alpha lipoic acid and 100, 150, 200, 250 and 300 µg/ml for L-Glutathione reduced were prepared from working standard solution. Prepared solutions were analyzed as per the proposed method. Five replicate analyses were carried out. The mean absorbance with its standard deviation and % relative standard deviation were calculated for both the drugs. Mean absorbance against concentration were plotted to obtain the calibration curves. Regression equations, co- relation coefficients were computed from calibration curves.

Limit of detection (LOD) and Limit of quantification(LOQ): LOD and LOQ were calculated from the data obtained from the linearity studies. For each of the five replicate determinations, slope and y-intercept of the linearity plot was determined. Average of slope

(S) and standard deviation of the y intercept (σ) was computed. From these values, the parameters LOD and LOQ were determined using following equations (On the basis of response and slope of the regression equation)

$$\text{LOD} = 3.3 \sigma/S$$

$$\text{LOQ} = 10 \sigma/S$$

Where; σ = Standard deviation of Response,

S = Slope of calibration curve

Precision

Repeatability: Six replicate solutions of sample equivalent to 40 $\mu\text{g}/\text{ml}$ Alpha lipoic acid and 200 $\mu\text{g}/\text{ml}$ L-Glutathione reduced were prepared. Prepared Solutions were analysed as per the proposed method. The mean % labelled claim with its standard deviation and % relative standard deviation were computed.

Intermediate precision: Intra-day precision and Inter-day precision were determined in terms of % RSD. Intraday precision was determined by analysing Alpha lipoic acid and L-Glutathione reduced for three times in the same day. Interday precision was hence determined by analysing Alpha lipoic acid and L-Glutathione reduced in sample solution at three independent concentration level of their respective calibration range for three days.

Intraday Precision: Replication within same day at different time: Sample solution containing mixture of Alpha lipoic acid and L-Glutathione reduced as 32 and 160 $\mu\text{g}/\text{ml}$, 40 and 200 $\mu\text{g}/\text{ml}$ and 48 and 240 $\mu\text{g}/\text{ml}$ in sample were analyzed for 3 times on the same days and %RSD were calculated.

Accuracy: Accuracy was calculated by addition of standard drug to pre-analyzed sample at 3 different concentration level and computing percentage recoveries. Accuracy was assessed using 9 determinations over 3 concentration levels covering the specified range (e.g., 3 concentrations and 3 replicates each of the total analytical procedure). Prepared solutions were analyzed as per the proposed method to have final level of 80%, 100% and 120% and % recoveries were calculated by proposed method. The mean percentage recovery with its standard deviation and % relative standard deviation were computed at each level.

Analysis of pharmaceutical formulation: Average weight of 20 tablets were taken. Tablets were triturated to powder. Powder equivalent to 50 mg L-Glutathione reduced and 10 mg

Alpha lipoic acid was transferred into 10 ml volumetric flask. Solution was further diluted with ethanol to obtain final concentration of 500µg/ml L-Glutathione reduced and 100µg/ml Alpha lipoic acid. From this, 0.4 ml was transferred to a 10 ml volumetric flask, 1ml of prepared Dichlone solution was added then diluted up to mark with ethanol. Solution was sonicated for 15 minutes and was filtered through Whatman filter paper No. 41. The absorbance of prepared sample solution i.e. A1 and A2 were recorded at 365 nm and 430 nm respectively. The analysis procedure was repeated six times. Relative concentration of two drugs in the sample was calculated using equation (1) and (2). The % stated value of the drugs was calculated.

$$C_x = \frac{A_1}{a_{x1} * b} \quad (1)$$

$$C_y = \frac{A_2 - a_{y2} C_x}{a_{y2}} \quad (2)$$

Where,

C_x = Concentration of Alpha lipoic acid

C_y = Concentration of L-Glutathione reduced

λ₁ = 365 nm (λ_{max} of Alpha lipoic acid),

λ₂ = 430 nm (λ_{max} of L-Glutathione reduced),

A₁ = absorbance of mixture at 365 nm

A₂ = absorbance of mixture at 430 nm,

a_{x1} = absorptivity of L-Glutathione reduced at 365 nm,

a_{x2} = absorptivity of L-Glutathione reduced at 430 nm,

a_{y2} = absorptivity of Alpha lipoic acid at 365 nm.

RESULTS AND DISCUSSION

Selection of suitable common solvent for solubility of both drugs

From the review of literature, it is concluded that both Alpha lipoic acid and L-Glutathione reduced are soluble in water and ethanol.

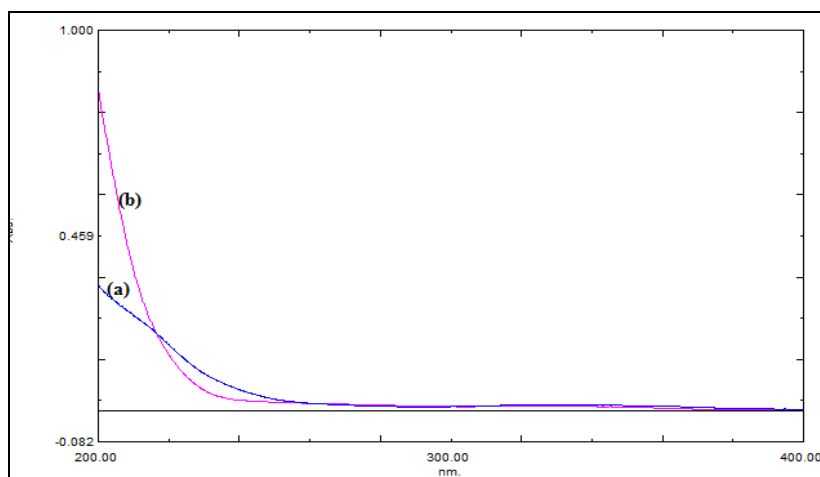


Fig. 3: Zero order overlaid spectra of (a) Alpha lipoic acid (10µg/ml) and (b) L-Glutathione reduced (50µg/ml) in water.

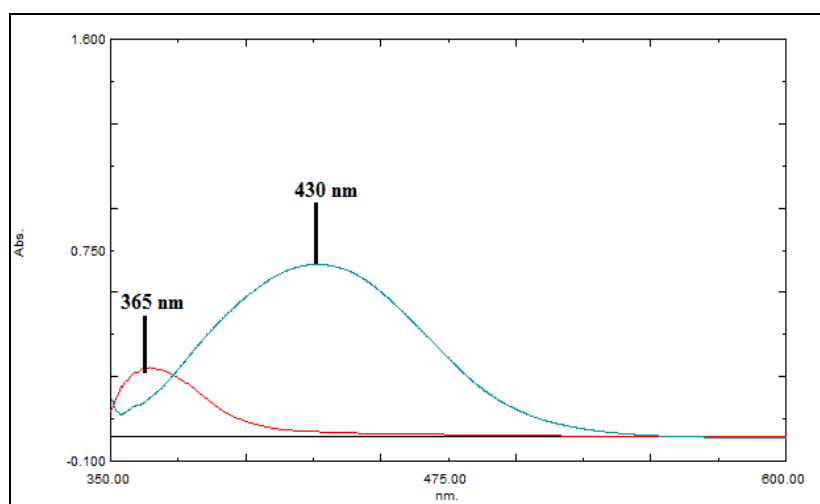


Fig. 4: Zero order overlaid spectra of Alpha lipoic acid (10µg/ml) and L-Glutathione reduced (50µg/ml) in water after derivatization with Dichlone reagent.

Using derivatization, both the drugs were better overlaid in the visible region.

Selection of Suitable Wavelength for Analysis

From the overlain spectra of Alpha lipoic acid and L-Glutathione reduced (Figure 6.9) 365 nm λ_{max} of Alpha lipoic acid (λ_1) and 430 nm λ_{max} of L-Glutathione reduced (λ_2) were selected as suitable wavelength for analysis of both drugs in Absorbance correction spectroscopy method.

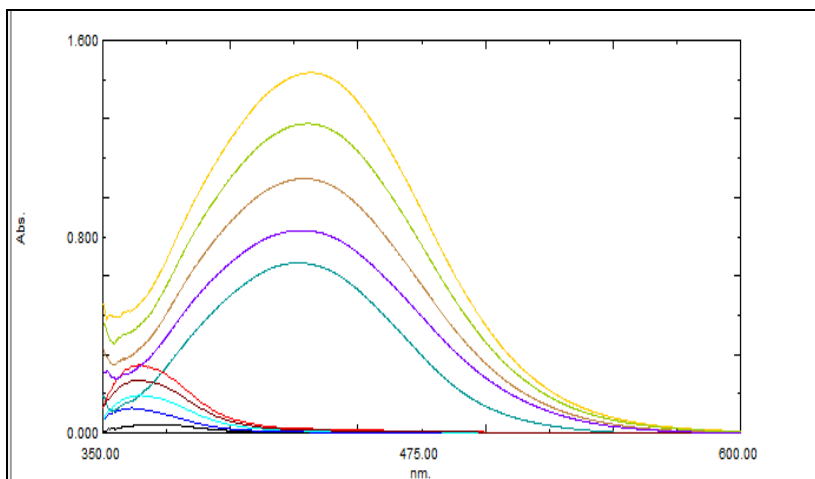


Figure. 5: Overlaid zero order spectra of Alpha lipoic acid (20-60µg/ml) and L-Glutathione reduced (100-300µg/ml).

Table. 1: Linearity Data of Alpha lipoic acid at 365 nm for Absorbance correction Method.

Sr. no.	Concentration (gm/100ml)	Absorbance*± SD	%RSD
1	0.002	0.0309±0.0004	1.5854
2	0.003	0.0959±0.0006	0.6916
3	0.004	0.153±0.0006	0.4133
4	0.005	0.2128±0.0007	0.3516
5	0.006	0.2756±0.0004	0.1777

* n=5

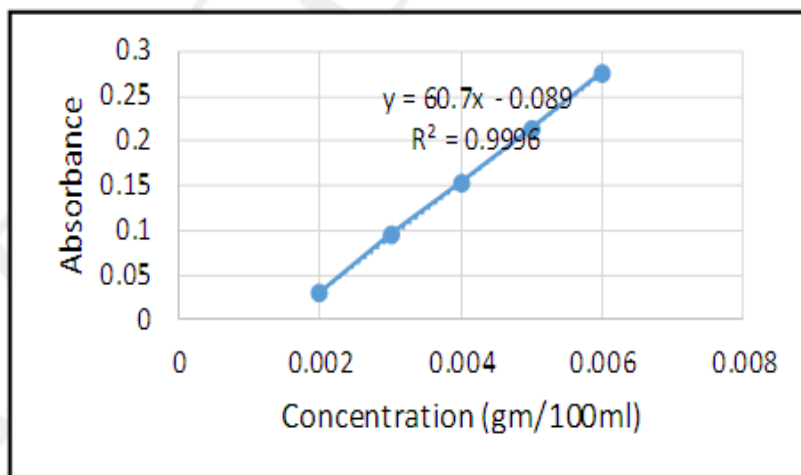


Fig. 6: Calibration curve of Alpha lipoic acid at 365 nm for Absorbance Correction method.

Table. 2: Linearity Data of L-Glutathione reduced at 365 nm for Absorbance correction Method.

Sr. no.	Concentration (gm/100ml)	Absorbance*± SD	%RSD
1	0.01	0.1574±0.0004	0.3152
2	0.015	0.2649±0.0006	0.2370
3	0.02	0.3533±0.0003	0.1049
4	0.025	0.4444±0.0003	0.0717
5	0.03	0.5334±0.0004	0.0816

*n=5

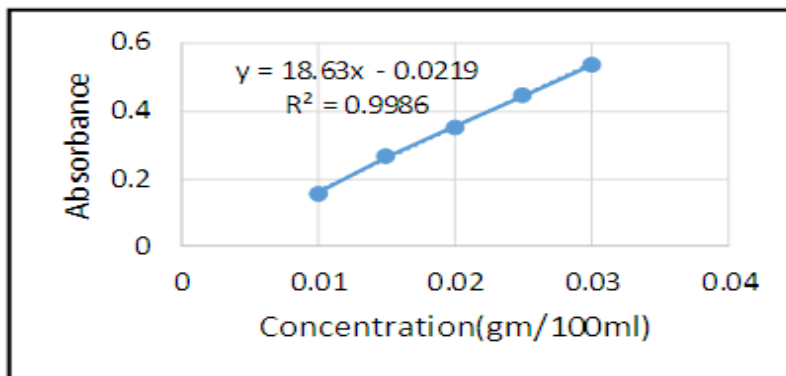


Fig. 7: Calibration curve of L-Glutathione reduced at 365 nm for Absorbance Correction method.

Table. 3: Linearity Data of L-Glutathione reduced at 430 nm for Absorbance Correction Method (*n=5).

Sr. no.	Concentration (gm/100ml)	Absorbance*± SD	%RSD
1	0.01	0.6890±0.0017	0.2596
2	0.015	0.8330±0.0018	0.2277
3	0.02	1.0804±0.0010	0.0943
4	0.025	1.2558±0.0039	0.3120
5	0.03	1.4666±0.0010	0.0701

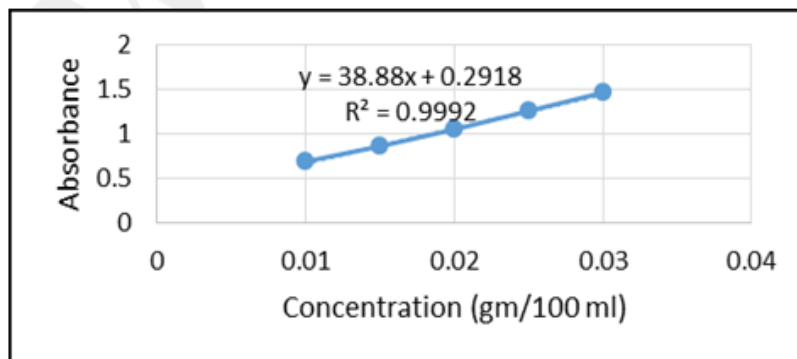


Fig. 8: Calibration curve of L-Glutathione reduced at 430 nm for Absorbance Correction method.

Table. 4: Result of Calibration curves of Alpha lipoic acid and of L-Glutathione reduced by Absorbance correction method.

Parameter		Alpha lipoic acid	L-Glutathione reduced
Regression equation	$\lambda_1 = 365 \text{ nm}$	$y = 60.7x - 0.089$	$y = 18.63x - 0.0219$
	$\lambda_2 = 430 \text{ nm}$	-	$y = 38.88x + 0.2918$
Correlation coefficient	$\lambda_1 = 365 \text{ nm}$	0.9996	0.9986
	$\lambda_2 = 430 \text{ nm}$	-	0.9992

Table. 5: Absorptivity values of Alpha lipoic acid and L-Glutathione reduced for Absorbance correction method

Drug	Absorptivity	
	$\lambda_1 = 365 \text{ nm}$	$\lambda_2 = 430 \text{ nm}$
Alpha lipoic acid	$a_{x1} = 60.7$	-
L-Glutathione reduced	$a_{y1} = 18.63$	$a_{y2} = 38.88$

Table. 6: Result of Range, Linearity, LOD and LOQ study for Absorbance correction method.

Parameter		Alpha lipoic acid	L-Glutathione reduced
Range		20 - 60 $\mu\text{g/ml}$	100 - 300 $\mu\text{g/ml}$
Linearity equation	$\lambda_1 = 365 \text{ nm}$	$y = 60.7x - 0.089$	$y = 18.63x - 0.0219$
	$\lambda_2 = 430 \text{ nm}$	-	$y = 38.88x + 0.2918$
Regression coefficient		0.9996	0.9986
		-	0.9992
LOD ($\mu\text{g/ml}$)		4.3660	2.1436
LOQ ($\mu\text{g/ml}$)		13.2304	6.4958

Table. 7: Repeatability study for Absorbance correction method.

Concentration ($\mu\text{g/ml}$)		Absorbance (n=6)	
Alpha lipoic acid	L-Glutathione reduced	Alpha lipoic acid	L-Glutathione reduced
40	200	0.4070	1.034
40	200	0.4065	1.033
40	200	0.4071	1.034
40	200	0.4069	1.035
40	200	0.4070	1.033
40	200	0.4068	1.032
Mean absorbance		0.4068	1.0335
SD		0.0001	0.0009
%RSD		0.0479	1.8500

Table. 8: Result of intraday precision for Absorbance correction method:

Conc. ($\mu\text{g/ml}$)		Mean Absorbance* \pm SD		%RSD	
Alpha lipoic acid	L-Glutathione reduced	Alpha lipoic acid	L-Glutathione reduced	Alpha lipoic acid	L-Glutathione reduced
32	160	0.0749 \pm 0.0001	0.5403 \pm 0.0012	0.1888	0.2308
40	200	0.1539 \pm 0.0001	1.0806 \pm 0.0012	0.0810	0.1154
48	240	0.2906 \pm 0.0103	1.1583 \pm 0.0151	0.0811	0.9571
Average % RSD				0.1169	0.4344

*n=3

Table. 9: Result of Interday precision for Absorbance correction method.

Conc. ($\mu\text{g/ml}$)		Mean Absorbance* \pm SD		%RSD	
Alpha lipoic acid	L-Glutathione reduced	Alpha lipoic acid	L-Glutathione reduced	Alpha lipoic acid	L-Glutathione reduced
32	160	0.0753 \pm 0.001	0.5720 \pm 0.0130	0.1998	0.2980
40	200	0.1840 \pm 0.001	1.203 \pm 0.0140	0.1988	0.1170
48	240	0.3020 \pm 0.0230	1.2030 \pm 0.0220	0.1321	0.999
Average % RSD				0.1769	0.4713

*n=3

Table. 10: Result of accuracy of Alpha lipoic acid for Absorbance correction method.

Level of Recovery (n=3)	Amount Of sample Taken ($\mu\text{g/ml}$)	Amount of std added ($\mu\text{g/ml}$)	Total amount ($\mu\text{g/ml}$)	Recovery of Alpha lipoic acid	%Recovery	SD	%RSD
0%	24	0	24	-	-	-	-
Mean% recovery							
80%	24	8	32	31.99	99.96	0.0141	0.0141
				31.98	99.93		
				31.99	99.96		
Mean% recovery					99.95		
100%	24	16	40	39.89	99.72	1.0866	1.0967
				39.99	99.97		
				39.02	97.55		
Mean% recovery					99.08		
120%	24	24	48	47.97	99.93	0.0571	0.0571
				48.03	100.06		
				48.02	99.85		
Mean% recovery					99.91		

Table. 11: Result of accuracy of L-Glutathione reduced for Absorbance correction method.

Level of Recovery (n=3)	Amount Of sample taken (µg/ml)	Amount of std added (µg/ml)	Total amount (µg/ml)	Recovery of L-Glutathione reduced	%Recovery	SD	%RSD
0%	120	0	120	-	-	-	-
Mean% recovery							
80%	120	40	160	159.90	99.93	0.2940	0.2947
				158.97	99.35		
				160.02	100.01		
Mean% recovery					99.76		
100%	120	80	200	199.89	99.94	0.1902	0.1908
				198.99	99.49		
				199.23	99.61		
Mean% recovery					99.68		
120%	120	120	240	239.94	99.97	0.0205	0.0205
				240.05	100.02		
				240.01	100.00		
Mean% recovery					99.99		

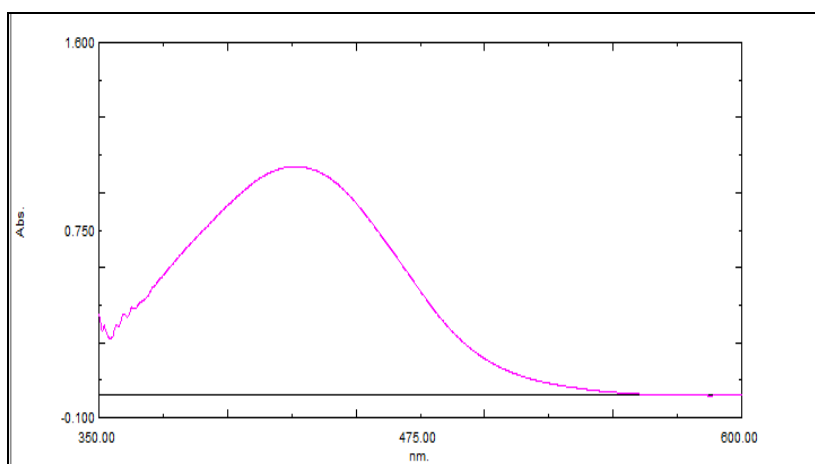


Fig. 9: Spectrum of sample by Absorbance correction method.

Table. 12: Analysis of Pharmaceutical formulation, (n=6).

Labelled claim(mg)		Amount obtained (mg)		% label claim	
Alpha lipoic acid	L-Glutathione reduced	Alpha lipoic acid	L-Glutathione reduced	Alpha lipoic acid	L-Glutathione reduced
100	500	99.93	498.67	99.93	99.73
100	500	99.96	498.12	99.96	99.62
100	500	98.80	500.02	98.80	100.0
100	500	99.99	496.01	99.99	99.20
100	500	100.01	489.02	100.01	97.80
100	500	99.98	498.03	99.98	97.80

Drug	Mean % Labelled claim (n=6)± SD	%RSD
Alpha lipoic acid	99.786±0.4459	0.4469
L-Glutathione reduced	99.793±0.1489	0.1492

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