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Simultaneous Estimation of Amlodipine and Atenolol by Using Reverse Phase High Performance Liquid Chromatography in Bulk and Marketed Formulation

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Abstract

A simple precise and accurate reverse phase high performance liquid chromatography (RP-HPLC) method has been developed for the simultaneous estimation of Amlodipine and Atenolol. Chromatographic separation was achieved on Altima C_{18} column (4.6×150mm, 5µ) using the mobile phase consisting of Methanol: TEA buffer: ACN (50:25:25 v/v). The mobile phase was pumped at a flow rate of 1.0 mL/min and detection was done by UV detector at 225 nm. The retention time for Amlodipine and Atenolol was 2.102 min and 3.577 min. The linearity concentrations for Amlodipine and Atenolol was 5-25 µg/ml and 12.5-62.5 µg/ml respectively with correlation coefficient was found to be 0.999. The proposed method was found to be simple, accurate, precise and cost effective for the simultaneous estimation of Amlodipine and Atenolol in pharmaceutical dosage forms.
Keywords: Amlodipine, Atenolol, RP-HPLC, Mobile phase, TEA Buffer

INTRODUCTION

Amlodipine belongs to the dihydropyridine (DHP) class of calcium channel blockers (CCBs), the most widely used class of CCBs. It decreases arterial smooth muscle contractility and subsequent vasoconstriction by inhibiting the influx of calcium ions through L-type calcium channels. Inhibition of the initial influx of calcium decreases the contractile activity of arterial smooth muscle cells and results in vasodilation[1,2]. Atenolol, a competitive beta (1)-selective adrenergic antagonist, has the lowest lipid solubility of this drug class. It competes with sympathomimetic neurotransmitter such as catecholamine's for binding at beta (1)-adrenergic receptors in the heart and vascular smooth muscle, inhibiting sympathetic stimulation. This results in a reduction in resting heart rate, cardiac output, systolic and diastolic blood pressure, and reflex orthostatic hypotension[3-5].

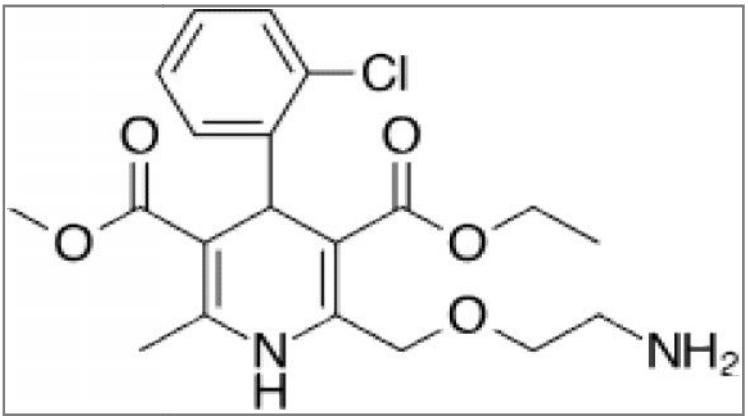

Fig 1: Structure of Amlodipine

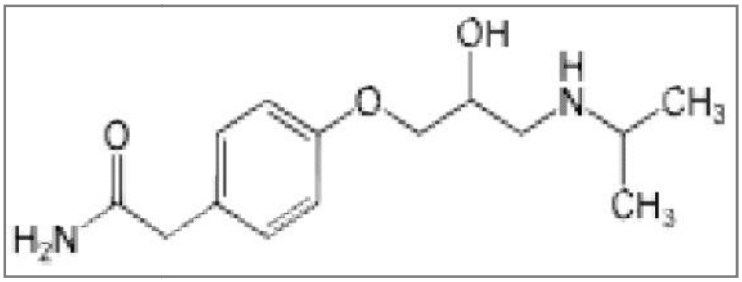

Fig 2: Structure of Atenolol

Literature survey reveals that few spectrophotometric and chromatographic methods have been reported for the estimation of Amlodipine and Atenolol in single and combination with other drugs[8-14]. Therefore an attempt has been made to develop and validate simple, precise and accurate RP-HPLC method for simultaneous estimation of Amlodipine and Atenolol in combined dosage form.

MATERIALS AND METHODS

Instrument used:

The liquid chromatographic system consists ofWaters HPLC with auto sampler and PDA Detector 996 model. Chromatographic analysis was performed on Altima C18 (4.6×150mm, 5µ) column.The analytes were monitored at 225 nm.

Materials used:

Working standards ofAmlodipine and Atenolol were procured as a gift sample by Sura labs, Hyderabad, India. Formulation of Amlodipine and Atenolol combined dosage form was purchased from local pharmacy.HPLC grade methanol, water and Acetonitrile was purchased from E. Merck (India) Ltd., Mumbai, India.AR Grade Tri ethyl amine buffer was obtained from S.D. Fine Chemicals Ltd., Mumbai, India.

Optimized Chromatographic Conditions:

Instrument used : Waters HPLC with auto sampler and PDA Detector 996 model.
Temperature : 40ºC
Column : Altima C18 (4.6×150mm, 5µ)
Buffer : Dissolve 1.5ml of Ttiethyl amine in 250 ml HPLC water and adjust the pH 4.5. Fliter and sonicate the solution by vaccum filtration and ultra sonication.
pH : 4.5
Mobile phase : Methanol: TEA buffer: ACN (50:25:25 v/v)
Flow rate : 1ml/min
Wavelength : 225 nm
Injection volume : 10 µl
Run time : 7 min

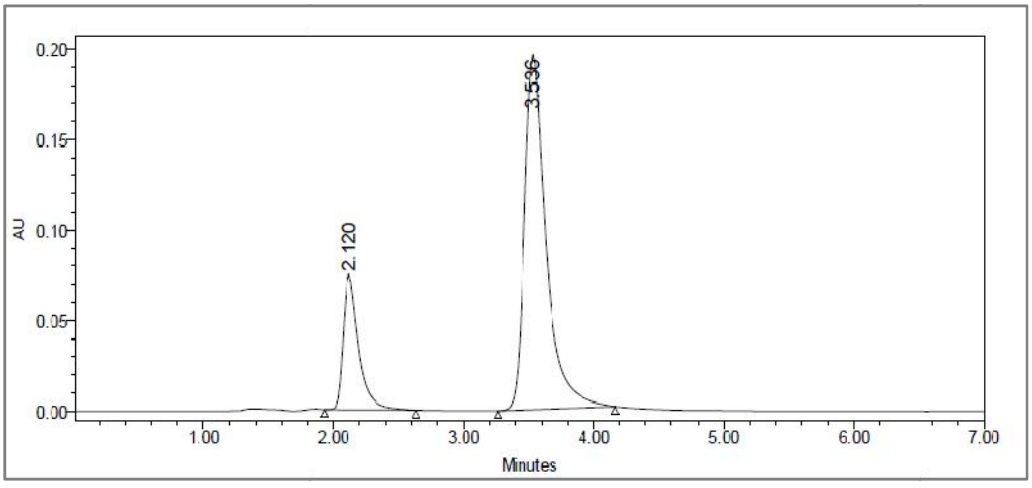

Fig 3: Optimized Chromatogram

Preparation of mobile phase:

Accurately measured 400 ml (40%) of Methanol, 200 ml of Triethyl amine buffer (20%) and 400 ml of Acetonitrile (40%) were mixed and degassed in digital ultra sonicater for 10 minutes and then filtered through 0.45 µ filter under vacuum filtration.

Preparation of Standard Solution:
Accurately weigh and transfer 10 mg of Amlodipine and 10mg of Atenolol working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution). Further pipettes 0.1ml of the above Amlodipine and 0.375ml of the Atenolol stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Preparation of Sample Solution:
Take average weight of one Tablet and crush in a mortor by using pestle and weight 10 mg equivalent weight of Amlodipine and Atenolol sample into a 10mL clean dry volumetric flask and add about 7mL of Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 0.1ml of the above Amlodipine and 0.375ml of the Atenolol stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

RESULTS AND DISCUSSION

Method validation
The developed analytical method was validated as per ICH guidelines for the parameters like specificity, linearity, accuracy, precision, robustness and system suitability.

System Suitability
Accurately weigh and transfer 10 mg of Amlodipine and 10mg of Atenolol working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution)**.** Further pipette 0.1ml of the above Amlodipine and 0.375ml of the Atenolol stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

Procedure:
The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limits. The results were shown in table 1.

Specificity
Inject the three replicate injections of standard and sample solutions and calculate the assay by using formula:

$$\%ASSAY = \frac{\text{Sample area}}{\text{Standard area}} \times \frac{\text{Weight of standard}}{\text{Dilution of standard}} \times \frac{\text{Dilution of sample}}{\text{Weight of sample}} \times \frac{\text{Purity}}{100} \times \frac{\text{Weight of tablet}}{\text{Label claim}} \times 100$$

The % purity of Amlodipine and Atenolol in pharmaceutical dosage form was found to be 99.6%.Results were shown in table 2 and fig 4,5.

Linearity
Accurately weigh and transfer 10 mg of Amlodipineand 10mg of Atenolol working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent. (Stock solution)

Preparation of Level – I (5 ppm of Amlodipine & 12.5ppm of Atenolol):
Pipette out 0.05ml of Amlodipine and 0.125ml of Atenolol stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – II (10 ppm of Amlodipine& 25ppm of Atenolol):
Pipette out 0.1ml of Amlodipine and 0.25ml of Atenolol stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – III (15 ppm of Amlodipine& 37.5ppm of Atenolol):
Pipette out 0.15 ml of Amlodipine and 0.375ml of Atenolol stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – IV (20 ppm of Amlodipine& 50ppm of Atenolol):
Pipette out 0.2 ml of Amlodipine and 0.5ml of Atenolol stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Preparation of Level – V (25 ppm of Amlodipine& 62.5ppm of Atenolol):
Pipette out 0.25ml of Amlodipine and 0.625ml of Atenolol stock solutions was take in a 10ml of volumetric flask dilute up to the mark with diluent.

Procedure:
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. Results were shown in table 3 and fig 6,7.

Precision
Preparation of Amlodipine and Atenolol Product Solution for Precision: Accurately weigh and transfer 10 mg of Amlodipine and 10mg of Atenolol working standard into a 10ml of clean dry volumetric flasks add about 7mL of

Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).Further pipette 0.1ml of the above Amlodipine and 0.375ml of the Atenolol stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent. The standard solution was injected for five times and measured the area for all five injections in HPLC. The %RSD for the area of five replicate injections was found to be within the specified limit. Results were shown in table 4.

Accuracy

For preparation of 50% Standard stock solution:

Accurately weigh and transfer 10 mg of Amlodipine and 10mg of Atenolol working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).Further pipette 0.075ml of the above Amlodipine and 0.187ml of the Atenolol stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 100% Standard stock solution:

Accurately weigh and transfer 10 mg of Amlodipine and 10mg of Atenolol working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).Further pipette 0.15ml of the above Amlodipine and 0.375ml of the Atenolol stock solutions into a 10ml volumetric flask and dilute up to the mark with Diluent.

For preparation of 150% Standard stock solution:

Accurately weigh and transfer 10 mg of Amlodipine and 10mg of Atenolol working standard into a 10ml of clean dry volumetric flasks add about 7mL of Diluents and sonicate to dissolve it completely and make volume up to the mark with the same solvent (Stock solution).Further pipette 0.225ml of Amlodipine and 0.56ml of Atenolol from the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluents.

Procedure:

Inject the Three replicate injections of individual concentrations (50%,100%,150%) were made under the optimized conditions. Recorded the chromatograms and measured the peak responses. Calculate the Amount found and Amount added for Amlodipine and Atenolol and calculate the individual recovery and mean recovery values. Results were shown in table 5.

Robustness

The analysis was performed in different conditions to find the variability of test results. The following conditions are checked for variation of results. .

Effect of Variation of flow conditions:

The sample was analyzed at 0.9 ml/min and 1.1 ml/min instead of 1ml/min, remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded.

Effect of Variation of mobile phase organic composition:

The sample was analyzed by variation of mobile phase i.e. Methanol: TEA Buffer: Acetonitrile was taken in the ratio and 40: 40:20, 60:10:30 instead (50:25:25), remaining conditions are same. 10µl of the above sample was injected and chromatograms were recorded. Results were shown in table 7.

Table 1: Results for System suitability

Sl.no.	Characteristic	Amlodipine	Atenolol
1.	Retention time (min)	2.102	3.577
1	Tailing factor	1.6	1.4
2	Theoretical plates	5377.2	5535.6
3	Resolution	2.02	
*Number of injections = Five replicates			

Table 2: Results for Specificity

S.No	Standard		Sample	
	Amlodipine	Atenolol	Amlodipine	Atenolol
1	607323	558777	775610	555592
2	606379	578377	689956	575685
3	606885	556966	607323	558777
Assay (% Purity)	99.6			

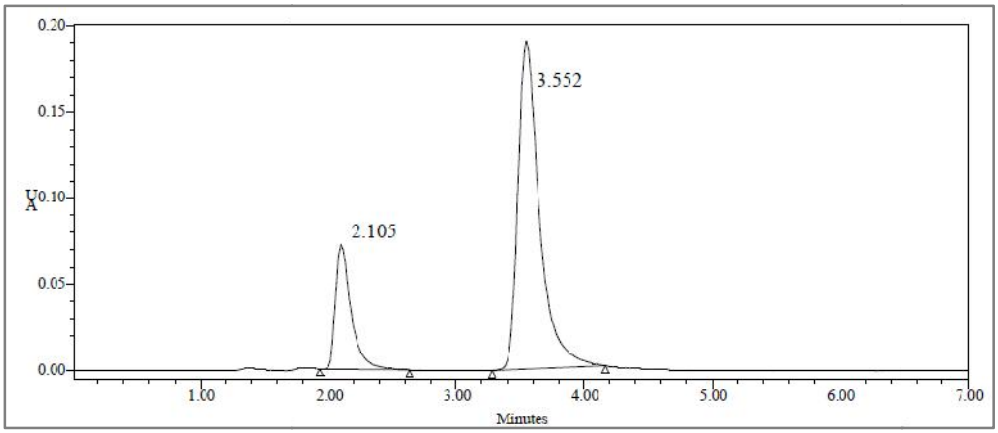

Fig 4: Chromatogram for Standard

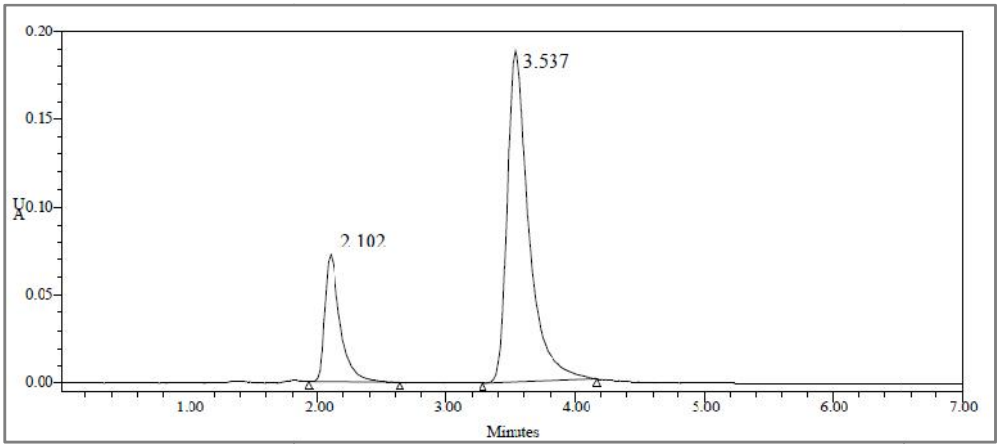

Fig 5: Chromatogram for Sample

Table 3: Results for linearity

Amlodipine		Atenolol	
Concentration(µg/ml)	**Peak area**	**Concentration(µg/ml)**	**Peak area**
5	205035	12.5	757881
10	381239	25	757881
15	561128	37.5	1458941
20	740162	50	2132457
25	909922	62.5	2901811

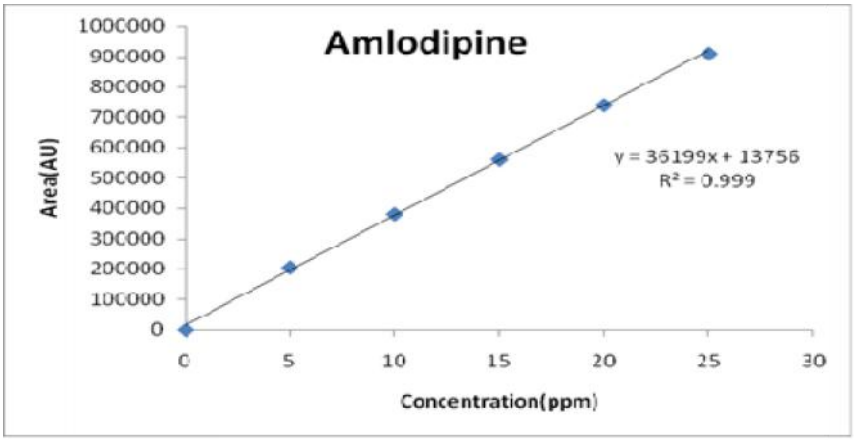

Fig 6: Calibration graph for Amlodipine

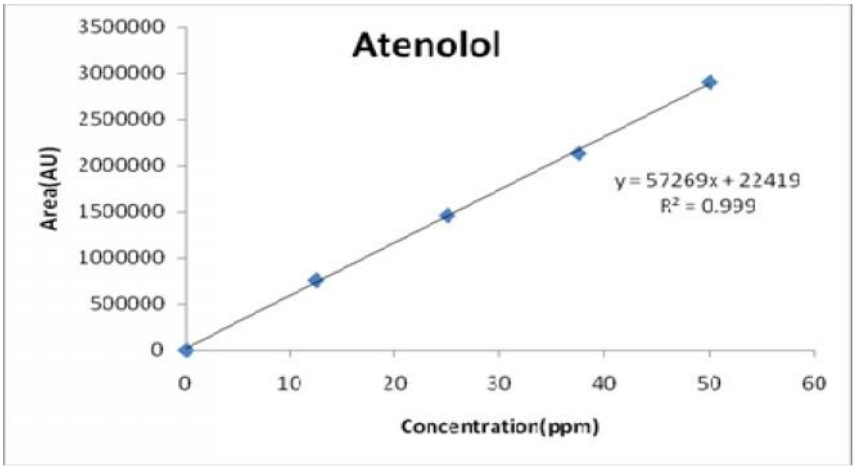

Fig 7: Calibration graph for Atenolol

Table 4: Results for Precision

	Amlodipine		Atenolol	
S.no	**Intra day**	**Inter day**	**Intra day**	**Inter day**
1	602223	596608	2220333	2207732
2	607748	598959	2221573	2202266
3	607302	595728	2215483	2209375
3	608674	594485	2217379	2204037
5	607376	595267	2211255	2204466
Avg	606665	596209	2217205	2205575
STD	2542.3	1718.7	4100.8	2899.8
%RSD	0.42	0.29	0.18	0.13

Table 5: Results for accuracy

Sample	Amount added (µg/ml)	Amount Recovered (µg/ml)	Recovery (%)	%Mean Recovery
Amlodipine	7.5	7.56	100.8	99.6%
Amlodipine	15	14.8	98.6	
Amlodipine	22.5	22.4	99.5	
Atenolol	18.75	18.73	100%	100%
Atenolol	37.5	37.4	99.9%	
Atenolol	56.25	56.21	100%	

Table 6: results for LOD @ LOQ

Drug	LOD(µg/ml)	LOQ(µg/ml)
Amlodipine	0.2	0.8
Atenolol	2.3	7.04

Table 7: Results for Robustness

Robust condition	Amlodipine			Atenolol		
	Retention Time	Theoretical plates	Tailing factor	Retention Time	Theoretical plates	Tailing factor
Actual Flow rate of 1.0 mL/min	2.102	5586	1.7	3.537	5371	1.6
Less Flow rate of 0.9 mL/min	2.330	5231	1.7	3.885	5324	1.7

More Flow rate of 1.1 mL/min	1.950	5234	1.7	3.263	5098	1.7
Less organic phase	2.290	5643	1.4	4.435	5239	1.2
More organic phase	1.998	5298	1.5	3.009	5647	1.0

Table 1: Analytical data of the Schiff base ligand and its mononuclear metal complexes

Compound	Molecular Formula	color	Yield %	Melting Point (º)	% of Nitrogen		% of Metal		Molar conductance Λ_m ($scm^2 mol^{-1}$)
					Cal	Exp	Cal	Exp	
L	$C_{16}H_{15}N_2OS$	Yellow	80	140	9.88	9.86	-	-	-
[CuLX]	$[Cu(C_{26}H_{22}N_4OS)]$	Dark Green	70	>200	11.15	11.14	12.64	12.63	132
[NiLX]	$[Ni(C_{26}H_{22}N_4OS)]$	Brown Black	75	>200	11.26	11.23	11.80	11.79	145
[CoLX]	$[Co(C_{26}H_{22}N_4OS)]$	Brown	70	>200	11.25	11.23	11.83	11.82	148

IR spectra

The IR spectra provide valuable information regarding the coordinating sites of Schiff base ligand which has been already discussed by Raman et al [9]. The IR spectra of the complexes were compared with that of the free ligand to determine the changes that might have taken place during the complexation. A comparative study of the IR spectra of ligand and its metal complexes reveals that certain peaks are common and therefore, only important peaks, which have either shifted or have newly appeared, are discussed. Table 2 shows that ν(C-O) and ν (C=N) modes occur at 1224-1332 cm^{-1} and 1595-1620 cm^{-1} respectively.

The shifting of (C-O) towards higher frequency as compared to the ligand (1224 cm^{-1}) is due to the conversion of hydrogen bonded structure into a covalent metal bonded structure. Lowering of (C=N) in the complexes as compared to the ligand (1620 cm^{-1}) is due to reduction of double bond character of carbon-nitrogen bond of the azomethine group. The band at 1440 cm^{-1} and 1522 cm^{-1} were due to symmetric stretching frequency and asymmetric frequency of acetate ion. This result predicts that the acetate ions were coordinated outside the coordination sphere. Metal-ligand bond is further confirmed by the appearance of a medium intensity band in the range 444-468, 402-410 and 530-560 cm^{-1} in the spectra of the complexes assigned to stretching frequencies of (M-N) bond, (M-S) bond and metal-oxygen bond formation respectively[10].

Table 2: Infrared Spectroscopic Data of the Schiff Base Ligand and its mononuclear metal complex

Compounds	(C=N) (cm^{-1})	(C-S) (cm^{-1})	(C-O) (cm^{-1})	(M−N) (cm^{-1})	(M−O) (cm^{-1})	(M−S) (cm^{-1})
$C_{16}H_{15}N_2OS$	1620	730	1244	--	--	--
$[Cu(C_{26}H_{22}N_4OS)]$	1605	710	1288	460	530	406
$[Ni(C_{26}H_{22}N_4OS)]$	1609	744	1320	454	544	410
$[Co(C_{26}H_{22}N_4OS)]$	1595	752	1332	468	560	408
$[Mn(C_{26}H_{22}N_4OS)]$	1600	718	1324	444	553	402

Electronic spectra and magnetic moment

The electronic spectral data of the metal complexes in DMF solution are displayed in Table 3. The nature of the ligand field around the metal ion was deduced from the electronic spectra. The electronic spectrum of Co(II) complex exhibited three bands in the region of 660, 570 and 530 nm which were tentatively assigned to $^4T1g \rightarrow 4T_2g(F)$ (v_1), $^4T_1g \rightarrow {}^4A_2g(F)$ (v_2) and $^4T_1g \rightarrow {}^4T_1g(P)$ (v_3) transitions, respectively. The value of magnetic moment was 5.12 B.M. which indicates the presence of Co(II) complex in octahedral geometry[11]. The electronic spectrum of the Ni(II) complex showed three bands at 695, 542 and 575 nm assignable to $^3A_2g \rightarrow {}^3T_2g$ (F) (v_1), $^3A_2g \rightarrow {}^3T_1g$ (F) (v_2) and $^3A_2g \rightarrow {}^3T_1g$ (P) (v_3) transitions, respectively. The value of magnetic moment was 3.42 B.M; therefore octahedral geometry is suggested for this complex[12].

The ^2Eg and ^2T_2g states of the octahedral Cu(II) (d^9) split under the influence of the tetragonal distortion and the distortion can be such as to cause the three transitions $^2B_1g \rightarrow {}^2B_2g$; $^2B_1g \rightarrow {}^2Eg$ and $^2B_1g \rightarrow {}^2A_1g$ to remain unresolved in the spectra. It is concluded that, all three transitions 690, 570 and 535 nm lie within the single broad envelope centered at the same range previously mentioned. This assignment is in agreement with the general observation that Cu(II) d–d transitions are normally close in energy. The magnetic moment of 1.97 B.M. falls within

the range normally observed for octahedral Cu(II) complexes [13]. The electronic spectra of Mn(II) complexes show the absorption bands in the range 694, 555 and 522 nm. These absorption bands may be assigned to the $^6A_1g \rightarrow {}^4A_1g$, $^6A_1g \rightarrow {}^4A_2g$, and $^6A_1g \rightarrow {}^4Eg$, ^4A_1g transitions, respectively. These bands suggest that the complexes possess an octahedral geometry. The Mn(II) complex show magnetic moments is 5.92 B.M. at room temperature corresponding to five unpaired electrons which suggest octahedral geometry[14]. In the spectra of the Schiff base ligand, the absorption band observed at 281-294 nm were assigned to intra-ligand $\pi \rightarrow \pi^*$ transition and the band at 342-390 nm were assigned due to $n \rightarrow \pi^*$ transition associated with the azomethine chromophore (-C=N).

Table 3: Electronic Spectral data of Schiff base ligand and its complexes.

Compound	Electronic spectra (nm)				Geometry of the complex
	$\pi \rightarrow \pi^*$	$n \rightarrow \pi^*$	$L \rightarrow M$	d-d	
$C_{16}H_{15}N_2OS$	294	386	-	-	-
$[Cu(C_{26}H_{22}N_4OS)]$	285	374	480	690,570,535	Octahedral
$[Ni(C_{26}H_{22}N_4OS)]$	292	390	440	695,542,575	
$[Co(C_{26}H_{22}N_4OS)]$	281	342	445	660,570,530	
$[Mn(C_{26}H_{22}N_4OS)]$	293	346	430	694,555,522	

^{1}H NMR spectra

The ^{1}H NMR Schiff base was recorded in DMSO- d_6 at room temperature. Three different type of protons were identified i) resonance exhibits due to phenolic –OH protons around 10.26 ppm , ii) characteristic resonance due to azomethine proton in the Schiff base appears at 8.227 ppm was observed and iii) the other signals in the region 6.24–7.72 ppm exhibits due to aromatic protons. All these observations support the infrared conclusions.

EPR spectra

The EPR spectra of complexes provide information of importance in studying the metal ion environment. The EPR spectra of the $[Cu(C_{26}H_{22}N_4OS)]$ Schiff base complexes recorded on powder samples with room temperature, on X-band at frequency 9.3 GHz under the magnetic field strength 4000 G. The EPR spectrum of the $[Cu(C_{26}H_{22}N_4OS)]$ (Figure 2) complexes show a broad signal with g_{iso} at 1.9998 which is consistent with an octahedral geometry [15].

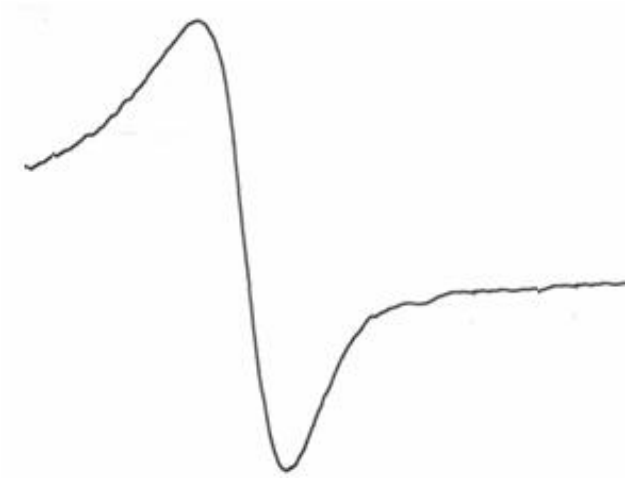

Figure.2.EPR spectra of Cu ($C_{26}H_{22}N_4OS$)] complex

CONCLUSION

In the present investigation, a simple, sensitive, precise and accurate RP-HPLC method was developed for the quantitative estimation of Amlodipine and Atenolol in bulk drug and pharmaceutical dosage forms. This method was simple, since diluted samples are directly used without any preliminary chemical derivatisation or purification steps. Amlodipine and Atenolol was freely soluble in ethanol, methanol and sparingly soluble in water. Methanol: TEA Buffer pH 4.5: Acetonitrile (50:25:25) was chosen as the mobile phase. The solvent system used in this method was economical. The %RSD values were within 2 and the method was found to be precise. The results expressed in Tables for RP-HPLC method was promising. The RP-HPLC method is more sensitive, accurate and precise compared to the Spectrophotometric methods. This method can be used for the routine determination of Amlodipine and Atenolol in bulk drug and in Pharmaceutical dosage forms.

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