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Research article

DEVELOPMENT OF VALIDATED UV SPECTROPHOTOMETRIC AND RP - HPLC METHOD FOR THE SIMULTANEOUS ESTIMATION OF AMBROXOL HYDROCHLORIDE AND DOXOFYLLINE FROM BULK DRUGS

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ABSTRACT

Ambroxol hydrochloride and Doxofylline are having π electrons in their structure and hence absorbs electromagnetic radiation in the UV region. This character of the drugs are used for estimating them in combination by UV-spectroscopic method using Jasco V-530 double beam UV-Vspectrophotometer, The calibration curves were obtained for both Ambroxol hydrochloride and Doxofylline in the range of 5-50µg/ml and 40-400µg/ml, respectively. The slope, intercept and correlation coefficient values of Ambroxol hydrochloride and Doxofylline at 249nm were found to be 0.0221, 0.0187 and 0.9983 and 0.0003, 0.0250, and 0.9996at 275nm were found to be 0.0019, 0.0137 and 0.9977 and 0.0032, -0.0014 and 0.9995, respectively. In RP-HPLC Ambroxol hydrochloride and Doxofylline system gave symmetric peak shapes, and minimum of tailing. The retention time for Ambroxol hydrochloride and Doxofylline were found to be 2.4 and 3.7 minutes, respectively. Linearity of Ambroxol hydrochloride and Doxofylline were found to be 1-10 µg/ml for both drugs and correlation coefficient value was found to be 0.999 and 0.997, respectively showing good correlation between concentration and peak area response. The LOD and LOQ of Ambroxol hydrochloride and Doxofylline were found to be 50 and 500 ng/ml respectively for both drugs.

Keywords: Ambroxol hydrochloride, Doxofylline, Correlation Coefficient, Electromagnetic radiation.

INTRODUCTION

UV spectroscopy is generally used in analysis for the quantitative determination of different analytes, such as transitionmetalions, biological macromolecules and highly conjugated organic compounds [1]. Spectroscopic analysis is commonly carried out in solutions but solids and gases may also be studied. Among the various spectrophotometric methods available, the technique of ultraviolet-visible spectroscopy is the most common method used in the analytical techniques used in the field of pharmacy [2]. It deals with the determination of the amount of UV (190-380 nm) or visible (380-800 nm) radiation absorbed by a substance in solution. High performance liquid chromatography is a convenient separation technique used for wide types of samples, with exceptional resolving power, speed and nano molecular detection levels [3]. This technique is based on the same modes of separation as that of classical chromatography such as adsorption, partition, ion exchange and gel permeation, but it differs from column chromatography in the fact that the mobile phase is passed through the packed column under high pressure [4]. Ambroxol chemical called trans-4-(2-Amino-3,5-dibrombenzylamino) cyclohexanol, it is yellow to yellowish crystalline powder, Soluble in water, acetonitrile, methanol and Alcohols. Whereas Doxofylline 7-(1,3-dioxolan-2-ylmethyl)- 1,3-dimethylpurine-2,6-

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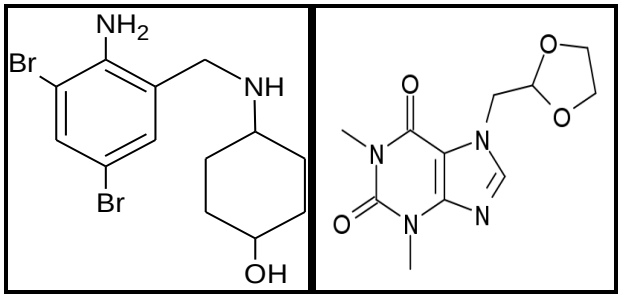
dione, it is White crystalline powder, Soluble in water, acetone, benzene, chloroform[5].



MATERIAL AND METHODS

Chemicals and solvents

- Methanol AR grade, HPLC grade (Qualigens Fine ChemicalsPvt. Ltd., Mumbai)
- Acetonitrile, HPLC grade (Merck Pvt.Ltd., Mumbai)

Materials used

- LichroCART C_{18} (250×4.0 mm, 5 µm) column (Merck Pvt. Ltd., Mumbai)
- Shimadzu HPLC System - Class LC 10 VP System with photodiode array detector was used for the study

Instruments Used

- Jasco V-530 UV/Vis Spectrophotometer

UV Spectrophotometric Method

Selection of solvent

Solubility of drugs were checked in solvents like water, methanol, ethanol, acetonitrile, etc. Absorbance of both drugs was higher and exhibited distinct λ_{max}in methanol and hence methanol was selected as solvent for further studies [6].

Selection of wavelength

A stock solution of 50µg/ml of Ambroxol hydrochloride and 400µg/ml of Doxofylline were prepared in methanol separately. These solutions were scanned in UV-region and their overlain spectra were recorded. The wavelength was selected as 249nm and 275nm.Different concentration of Ambroxol hydrochloride (5-50µg/ml), Doxofylline (40-400µg/ml) and mixture of Ambroxol hydrochloride and Doxofylline were prepared from respective stock solutions [7]. The absorbance was noted at the above said wavelengths, good linearity was observed hence these wavelengths were selected for their simultaneous estimation. The absorptivity was calculated for Ambroxol hydrochloride and Doxofylline at the selected wavelengths and the values are shown in table 1&2.

Method Validation by UV

The developed method was validated in terms of parameters like precision, linearity and stability studies [8].

Linearity and range

Ambroxol hydrochloride

Ambroxol hydrochloride was found to be linear in the concentration range of 5-50 µg/ml. The absorbance of these solutions were noted at the selected wavelengths, 249nm and 275nm, and calibration curves were plotted using concentration and absorbance. At wavelength 249nm-slope, intercept and correlation coefficient values were found to be 0.0221, 0.0187 and 0.9983 and at 275nm-slope, intercept and correlation coefficient values were found to be 0.0019, 0.0137 and 0.9977.

Doxofylline

Doxofylline was found to be linear in the concentration range of 40-400 µg/ml. At wavelength 249nm-slope, intercept and correlation coefficient values were found to be 0.0003, 0.0250, and 0.9996 and at 275nm- slope, intercept and correlation coefficient values were found to be 0.0032, -0.0014 and 0.9995.

Precision

Precision of the method was demonstrated by

Intraday precision

Intraday precision was found out by carrying out the analysis of the standard drug for three different concentrations in the linearity range of drug for three times on the same day and %RSD was calculated as shown in table 3.

Inter day precision

Inter day precision was found out by carrying out the analysis of the standard drug for three different concentrations in the linearity range of drug for three days over a period of one week and %RSD were calculated shown in table 4.

Stability Studies

When the prepared solutions are exposed to atmosphere, the analytes are likely to decompose. Hence it is necessary to conduct stability studies. Stability of the analytes in the solutionswas studied at different time intervals and absorbance was compared with the absorbance of freshly prepared solution. The solutions were found to be stable for about 7 hours as reduction of absorbance was within the limits in table 5.

Recovery Studies

In order to ensure the suitability and reliability of proposed method, recovery studies were carried out. To an equivalent quantity of mixture, a known quantity of standard Ambroxol hydrochloride and Doxofylline

were added at 50 and 100% level and the contents were re-analysed by the proposed method. The % recovery and %RSD were calculated in table 6.

RP-HPLC Method

Selection of mobile phase

Solvent selectivity (solvent type), solvent strength (percentage of organic solvent in the mobile phase), strength and pH of buffer, flow rate etc. were varied to determine the chromatographic conditions that gave the best separation in table 7.

Selection of Ratio of Mobile phase

In a mobile phase system consisting water (pH-3.4) and acetonitrile in different ratios like 40:60, 30:70 and 50:50 %v/v, a mixture of Ambroxol hydrochloride and Doxofylline were injected. Symmetrical peaks with good resolution was obtained with a ratio of 40:60 %v/v and hence selected for further studies [9].

Selection of flow rate

Keeping all the parameters of mobile phase system constant, the chromatograms were recorded with different flow rates like 0.6, 0.8 and 1ml/ min. with flow rate 0.6 and 1ml/ min, peaks were not symmetrical. But a flow rate of 0.8ml/min gave good symmetrical peaks and hence selected for further studies [10].

Method Validationby RP-HPLC

Limit of detection (LOD) and Limit of quantification (LOQ)

LOD and LOQ were determined by injecting progressively lower concentrations of the drug. LOD of Ambroxol hydrochloride and Doxofylline were found to be 50ng/ml and LOQ of Ambroxol hydrochloride and Doxofylline were found to be 500ng/ml.

Linearity and range

Calibration graph was plotted using standard drug peak areas Vs. concentration of standard solutions table 8. Linear regression data revealed an excellent linear relationship for both the drugs in the concentration range of 1-10µg/ml. The slope, intercept and correlation co-efficient values were found to 22979.8485, 2071.7333 and 0.999113 respectively, for Ambroxol hydrochloride and 35314.9879, -5270.7333 and 0.997979 respectively, for Doxofylline [11].

Chromatogram of Standards

Precision

Intraday precision

Intraday precision was done by carrying out analysis of standard drugs solutions at a concentration in the linearity range (5µg/ml) for six times on the same day and %RSD was calculated in table 9.

Inter day precision

Inter day precision was done by carrying out the analysis of standard drugs solutions at a concentration in the linearity range (5µ/ml) for three days and %RSD was calculated in table 10.

Accuracy

Recovery studies were done for determining accuracy parameter. It was done by mixing known quantity of standard drug with the analysed sample mixture and the contents were reanalyzed by the proposed method.Recovery studies carried out at 50 and 100% levels [12]. The percentage recovery and its %RSD were calculated table 12.

Repeatability of injection

A standard solution of drugs (5µg/ml) was injected 6 times and its %RSD was calculated in table 11.

Stability

Sample solution of Ambroxol hydrochloride and Doxofylline were subjected to stability studies under refrigerated and room conditions. Stabilities were studied by looking for any change in retention time, resolution, peak shape, etc. when compared to chromatogram of freshly prepared solution, the solution stored under room temperature was stable up to 16 hours and under refrigeration up to 48 hours [13].

System suitability studies

System suitability parameters like number of theoretical plates (N), peak asymmetry factor (As), resolution (Rs) etc. were studied (table 37).

Robustness

In order to demonstrate the robustness of the method, the following optimized conditions were slightly varied.

- ± 1 in ratio of methanol in mobile phase
- ± 0.1 units in pH of buffer
- ± 0.1 in flow rate

The response factors for these changed chromatographic parameters were almost same as that of the fixed chromatographic parameters and hence developed method is said to be robust in table 14.

Specificity

Conditions of HPLC method like percentage of organic solvent in mobile phase, ionic strength, pH of buffer, flow rate etc, were changed. Although these changes were made, no additional peaks were found but there were some slight changes in retention times and peak shapes. Peak purity tests were done. The peak purity index of Ambroxol hydrochloride and Doxofyllinewere found to be 1.0000 and 1.0000, respectively. Peak purity index values close to one proves peak purity of the drugs.

ANALYSIS OF MIXTURE

Fixed chromatographic conditions were made use for the analysis of mixture.

Preparation of standard solutions

Stock solution of Ambroxol hydrochloride (100µg/ml) and Doxofylline (100µg/ml) were prepared in methanol.

Preparation of mixture

A quantity of 30mg of Ambroxol hydrochloride and 400mg of Doxofylline were weighed, transferred to a 100ml standard flask and made up to volume with methanol. This solution was further diluted to get the concentration under the range of linearity.

Recording of chromatogram

A steady baseline was recorded with the fixed chromatographic conditions. Standard drugs solutions (1 to 10µg/ml of Ambroxol hydrochloride and 1 to 10µg/ml of Doxofylline) were injected and chromatograms were recorded. Retention time of Ambroxol hydrochloride and Doxofylline were found to be 2.4 and 3.7 minutes, respectively. This was followed by injection of sample solution obtained from mixture [14].

Calibration curves were plotted using peak area of standard drugs Vs concentration of standard solutions. Peak areas of the sample chromatograms were compared and amount of Ambroxol hydrochloride and Doxofylline were calculated in table 15.

Table 1. Absorbance and absorptivity of Ambroxol hydrochloride at selected wavelengths

Conc. (µg/ml)	249nm			275nm		
	Abs.	Absorptivity	Avg. Absorptivity	Abs.	Absorptivity	Avg. Absorptivity
5	0.1274	0.02548	0.02317	0.0258	0.00516	0.00273
10	0.2447	0.02447		0.0338	0.00338	
15	0.3414	0.02276		0.0410	0.00273	
20	0.4614	0.02307		0.0504	0.00252	
25	0.5882	0.02352		0.0594	0.00237	
30	0.6495	0.02165		0.0675	0.00225	
35	0.7960	0.02274		0.0779	0.00221	
40	0.9267	0.02316		0.0910	0.00227	
45	1.0364	0.02303		0.1004	0.00223	
50	1.0944	0.02188		0.1096	0.00219	

Table 2. Absorbance and absorptivity of Doxofylline at selected wavelengths

Conc. (µg/ml)	249nm			275nm		
	Abs.	Absorptivity	Avg. Absorptivity	Abs.	Absorptivity	Avg. Absorptivity
40	0.0367	0.00091	0.00044	0.1506	0.00376	0.00321
80	0.0463	0.00057		0.2334	0.00291	
120	0.0564	0.00047		0.3782	0.00315	
160	0.0670	0.00041		0.5011	0.00313	
200	0.0765	0.00038		0.6424	0.00321	
240	0.0873	0.00036		0.7609	0.00317	
280	0.0984	0.00035		0.8907	0.00318	
320	0.1095	0.00034		1.0127	0.00316	
360	0.1200	0.00033		1.1435	0.00317	
400	0.1316	0.00032		1.2818	0.00320	

Table 3. Intraday precision

Ambroxol hydrochloride			Doxofylline		
Concentration (µg/ml)	Absorbance at 249nm	%RSD*	Concentration (µg/ml)	Absorbance at 275nm	%RSD*
10	0.2447	0.18	80	0.2334	0.97
	0.2390			0.2362	
	0.2481			0.2381	
30	0.6495	0.14	240	0.7609	0.28

	0.6573			0.7583	
	0.6424			0.7566	
50	1.0944	0.83	400	1.2818	0.71
	1.1129			1.2652	
	1.1022			1.2669	

*mean of three observations

Table 4. Inter day precision

Day	Ambroxol hydrochloride			Doxofylline		
	Concentration (µg/ml)	Absorbance at 249nm	%RSD*	Concentration (µg/ml)	Absorbance at 275nm	%RSD*
1	10	0.2447	0.99	80	0.2334	0.12
2		0.2473			0.2388	
3		0.2496			0.2346	
1	30	0.6495	0.34	240	0.7609	0.38
2		0.6456			0.7555	
3		0.6450			0.7601	
1	50	1.0944	0.14	400	1.2818	0.23
2		1.0771			1.2790	
3		1.1090			1.2759	

*mean of three observations

Table 5.Stability studies

Time (hrs)	Ambroxol hydrochloride		Doxofylline	
	Concentration (µg/ml)	Absorbance at 249nm	Concentration (µg/ml)	Absorbance at 275nm
0	30	0.6495	400	1.2818
1		0.6487		1.2714
2		0.6321		1.2675
3		0.6357		1.2427
4		0.6125		1.2237
5		0.6035		1.1925
6		0.5989		1.1812
7		0.5972		1.1695
8		0.5801		1.1522

Table 6. Recovery studies

Level	% Recovery		%RSD*	
	Ambroxol hydrochloride	Doxofylline	Ambroxol hydrochloride	Doxofylline
50%	99.87	100.65	0.160	0.321
100%	99.65	98.58	0.317	0.457

*****mean of six observations

Table 7. Selection of mobile phase

Mobile phase	Observation
Methanol : Water	Broad peaks
Acetonitrile : water	Good separation with tailing
Acetonitrile : water(pH-2.5)	Good separation with fronting
Acetonitrile : water(pH-3.4)	**Good separation with symmetrical peaks**
Acetonitrile : water(pH-4)	Good separation with tailing

Table 8. Calibration data of Ambroxol hydrochloride and Doxofylline

Concentration (µg/ml)	Peak area	
	Ambroxol hydrochloride	Doxofylline
1	25860	34360
2	48054	68887
3	70485	100326
4	95401	135796
5	114131	163013
6	139679	201863
7	164793	237036
8	187901	275109
9	202378	328553
10	235927	344674

Table 9. Intraday presicion

Concentration (µg/ml)	Peak area		%RSD*	
	Amboxol hydrochloride	Doxofylline	Ambroxol	Doxofylline
5	114131	163013	0.836	0.448
	114682	163454		
	115643	164455		
	116783	162999		
	114457	163838		
	115468	163912		

*mean of six observations

Table 10. Inter day presicion

Day	Concentration (µg/ml)	Peak area		%RSD*	
		Amroxol hydrochloride	Doxofylline	Ambroxol	Doxofylline
1	5	114131	163013	0.826	0.467
2		118663	164224		
3		118221	162299		

*mean of three observations

Table 11. Repeatability of injection

Concentration (µg/ml)	Injection	Peak area		%RSD*	
		Ambroxol hydrochloride	Doxofylline	Ambroxol hydrochloride	Doxofylline
5	1	118798	176280	0.6452	0.9234
	2	118653	176165		
	3	118773	176315		
	4	118810	176267		
	5	118642	176520		
	6	118321	176421		

*mean of six observations

Table 12. Recovery studies

Level	% Recovery		%RSD*	
	Ambroxol hydrochloride	Doxofylline	Ambroxol hydrochloride	Doxofylline
50%	99.86	101.36	0.219	0.354
100%	98.65	100.21	0.432	0.167

*****mean of six observations

Table 13. System suitability studies

Drugs	R_s	N	A_s	Tailing factor
Ambroxol hydrochloride	6.893	5989.484	1.379	1.244
Doxofylline		6210.504	1.351	1.195

Table 14. Robustness

Chromatographic condition		Peak area		Fig.no
		Ambroxol hydrochloride	Doxofylline	
Mobile phase ratio (Acetonitrile: Water pH-3.4)	59:41	114636	164183	71
	61:39	116397	163226	72
pH of Water	3.3	118798	176280	73
	3.5	120561	177727	74
Flow rate	0.7	132937	187570	75
	0.9	103709	146893	76

Table 15. Analysis of mixture

Drug	Amont of drug (mg)		Assay(%) ± %RSD*
	Amount taken	Estimated	
Ambroxol hydrochloride	30	29.74	99.13 ± 0.126
Doxofylline	400	400.86	100.22 ± 0.115

*mean of six observations

Table 16. Analysis of Ambroxol hydrochloride and Doxofylline in Bulk drug

Drug	UV Spectrophotometry			HPTLC			HPLC		
	Est. Amt (mg)	% RSD*	Linearity (µg/ml)	Est. Amt (mg)	% RSD*	Linearity (ng/band)	Est. Amt (mg)	% RSD*	Linearity (µg/ml)
Amb	30.94	0.17	5-50	29.97	0.2	300-1100	29.74	0.42	1-10
Dox	400.76	1.08	40-400	400.52	0.2	100-1100	400.86	0.31	1-10

*mean of six observations

SUMMARY AND CONCLUSION

UV Spectroscopic Method

Estimation of Ambroxol hydrochloride and Doxofylline was achieved by simultaneous equation method using Jasco V-530 double beam UV-Vis spectrophotometer. Stock solutions of Ambroxol hydrochloride and Doxofylline were prepared in methanol. The calibration curves were obtained for both Ambroxol hydrochloride and Doxofylline in the range of 5-50µg/ml and 40-400µg/ml, respectively. The slope, intercept and correlation coefficient values of Ambroxol hydrochloride and Doxofylline at 249nm were found to be 0.0221, 0.0187 and 0.9983 and 0.0003, 0.0250, and 0.9996, respectively. The slope, intercept and correlation coefficient values of Ambroxol hydrochloride and Doxofylline at 275nm were found to be 0.0019, 0.0137 and 0.9977 and 0.0032, -0.0014 and 0.9995, respectively.

The recovery studies were carried out to ensure the reproducibility and reliability of the method by adding known amount of standard drug solutions and analysis was carried as per developed procedure.

RP-HPLC Method

In RP-HPLC method, optimizations of different chromatographic parameters like selection of chromatographic method, detection wavelength, selection of mobile phase, ionic strength of mobile phase, mobile phase ratio, flow rate etc. were done. A wavelength of 255nm was selected for the study. A mobile phase system (binary mixture) containing water (pH-3.4) : acetonitrile (40:60, v/v), flow rate – 0.8 mL/min, was employed for the determination of Ambroxol hydrochloride and Doxofylline because this system gave symmetric peak shapes, and minimum of tailing. The retention time for Ambroxol hydrochloride and Doxofylline were found to be 2.4 and 3.7 minutes, respectively as shown in table 16.

Linearity of Ambroxol hydrochloride and Doxofylline were found to be 1-10 µg/ml for both drugs and correlation coefficient value was found to be 0.999 and 0.997, respectively showing good correlation

between concentration and peak area response. The LOD and LOQ of Ambroxol hydrochloride and Doxofylline were found to be 50 and 500 ng/ml respectively for both drugs. Precision of the developed method was studied under inter-day, intra-day and repeatability studies. A low relative standard deviation value shows that the developed method is precise. Recovery study was carried out at 50% and 100% levels. System suitability parameters were studied like number of theoretical plates (N), asymmetry factor (As), resolution (Rs) etc. were studied. The validated RP-HPLC method was applied to the determination of Ambroxol hydrochloride and Doxofylline in bulk drugs.

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Nil

CONFLICT OF INTEREST

Nil

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