Journal of Pharmaceutical and Scientific Innovation

www.jpsionline.com

Research Article

HPLC METHOD FOR THE DETERMINATION OF AMBROXOL HCL IN THE PRESENCE OF ANTIMICROBIAL PRESERVATIVES IN ORAL LIQUID FORMULATION

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DOI: 10.7897/2277-4572.04230

Received on: 25/01/15 Revised on: 16/02/15 Accepted on: 25/03/15

ABSTRACT

A simple gradient reversed phase high performance liquid chromatographic method was developed for the determination of ambroxol hydrochloride in the presence of antimicrobial preservatives in oral liquid formulation. The chromatographic separation was achieved on an Inertsil C_8 (250 X 4.6 mm 5 µ particle size) column using gradient elution at PDA detector. The optimized mobile phase consisted of 0.1 % Trifluoroacetic acid as a mobile phase A and a mixture of mobile phase A and acetonitrile in the ratio of (76:24 % v/v) as mobile phase B. The compounds were eluted at a flow rate of 1.0 ml/min. The method validated according to the International conference Harmonization (ICH) guidelines. The validation characteristics included accuracy, precision, linearity, specificity, limit of detection, limit of quantification and robustness. The calibration curves were linear over the ($r^2 > 0.99$) concentration range from 300 to 900 ppm for ambroxol hydrochloride, 10 to 30 ppm for propyl paraben and 100 to 300 ppm for methyl paraben. The percentage recoveries were found to be in the range from 99.55 to 101.1 % for ambroxol hydrochloride, 100.31 to 101.92 % for propyl paraben and 98.18 to 101.61 % for methyl paraben. Stability indicating capability was established by forced degradation experiments. No chromatographic interference from the degradation products was found. The proposed method was highly sensitive, precision and accurate and hence successfully applied for the quantification of ambroxol active pharmaceutical ingredients (API) and preservatives content in the commercially available oral liquid formulation.

Keywords: Ambroxol hydrochloride, methylparaben, propylparaben, method validation, forced degradation, assay.

INTRODUCTION

Ambroxol hydrochloride (AMB) chemically Trans-4-(2-Amino-3, 5-dibrombenzyl amino) cyclohexanol hydrochloride (Figure 1) is a semi-synthetic derivative of vasicine obtained from Indian shrub *Adhatoda vasica*. It is a metabolic product of bromhexine. It is used as broncho secretolytic and an expectorant drug[1]. It simulates the transportation of the viscous secretions in the respiratory organs and reduces the stand stillness of the secretions.

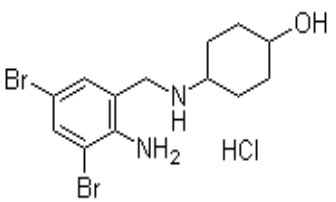

Figure 1: Ambroxol hydrochloride

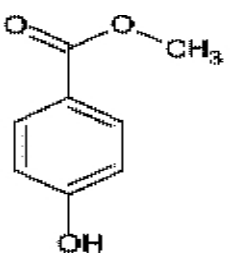

Figure 2: Methyl Paraben

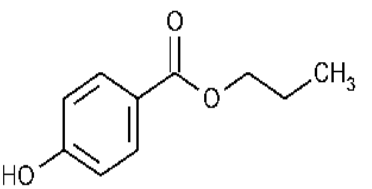

Figure 3: Propyl Paraben

Methylparaben (MP) chemically known as Methyl 4-hydroxybenzoate (Figure 2) and Propylparaben (PP) chemically known as Propyl 4-hydroxybenzoate (Figure 3) are used as either single or in combinations in drug products as antimicrobial preservatives to prevent alteration of product preparations. In most pharmaceutical preparations, especially in syrup a preservative is essential because the excipients and sometimes the drug itself may be destroyed by different microorganisms and consequently the formulation breaks down. Synthetic preservatives constitute the largest and most commonly used group in the preservatives of pharmaceutical products. The esters of P-hydroxy benzoic acid with different alcohols are known as hydroxybenzoate or parabens[2]. Several different methods have been reported for individual determination of AMB HCL in pharmaceutical preparations by spectrophotometry[3-7]. Simultaneous estimation of AMB with Cefpodoxime proxetile and Guiaphensin and their dosage form by UV spectrophotometric methods have been reported[8-9]. AMB determination in biological fluids by capillary electrophoresis with fluorescence detection[10] (Thomas) Gas Chromatography with electron capture detection[11] (Colombo) capillary gas liquid chromatography[12] (Schmid) HPLC with amperometric detection[13] (Flores) and LC-MS/MS[14] (Hohyun) AMB HCL combines with BA

(benzoic acid) in a syrup as pharmaceutical form stress test for stability evaluation by HPLC[15] (Maarit), AMB with MP and BA in pharmaceutical preparations based on sequential injection technique coupled with monolithic column[16], AMB with different preservatives in pharmaceutical formulations by HPLC[17] and RP-UPLC[18] methods have been reported. Simultaneous estimation of desloratadine hydrochloride and AMB, stability indicating method for the estimation of cetrizine hydrochloride and AMB HPTLC method have been reported[19-20]. However in the HPLC methods reported in the literature for the determination of AMB in oral liquid preparations, co - elution of AMB with only parabens or other excipients should not be considered. The current work describes, determination of AMB in oral liquid preparations containing only with two parabens (preservatives MP and PP). Market formulations containing only two parabens (MP and PP) have been selected for the analysis. Therefore it was thought worthwhile to develop, simple, precise and accurate HPLC method for the determination of AMB with two parabens without sample pretreatment.

MATERIALS AND METHODS

Materials and Reagents

Ambroxol hydrochloride, Marketed formulation, Placebo syrup, propyl and methyl parabens were kindly donated by Dr. Reddy's laboratories limited, Hyderabad, India. HPLC grade methanol and acetonitrile were obtained from Rankem, GR grade potassium hydrogen phosphate, ortho phosphoric acid and trifluro acetic acid were obtained from Merck limited, Mumbai, India.

Equipments

Cintex digital water bath was used for specificity study. Photo stability studies were carried out in a photo-stability chamber (SUNTEST XLS$^+$, ATLAS and Germany). Thermal stability studies were performed in a dry air oven (Cintex, Mumbai, India).

Chromatographic Conditions

Analyses were performed on HPLC system (Waters, Milford, USA), consisting of a quaternary solvent manager, sample manager and PDA (photo diode array) detector. System control, data collection and data processing were accomplished using Waters Empower TM-2 chromatography data software. The chromatographic condition was optimized using Inertsil C_8, 5 µm (250 mm x 4.6 mm) column. Solvent A consisted of 0.1 % trifluoroacetic acid and acetonitrile: solvent-A in the ratio of 76:24 (v/v) was used as a solvent-B. Solvents-A and B were degassed under vacuum prior to use. Mixture of water and methanol in the ratio of 50:50 (v/v) respectively was used as a diluent. The finally selected and optimized conditions were as follows: injection volume 20 µL, gradient elution (Table 1) at a flow rate of 1.0 mL/min at 50°C (column oven) temperature, detection wavelength 245 nm. Gradient programme was shown in Table 1.

Preparation of standard solution

Preparation of standard stock solution for Propyl paraben

40 mg of propyl paraben working standard was accurately weighed and transferred into a 100 ml volumetric flask. 50 ml of methanol was added, sonicated to dissolve and made up to volume with methanol and mixed well (20 ppm).

Preparation of standard stock solution for Methyl paraben and Ambroxol HCl

40 mg of MP working standard (200 ppm) and 120 mg of AMB HCL (600 ppm) were accurately weighed and transferred into a 200 ml volumetric flask. 100 ml of diluent was added and sonicated to dissolve. 10 ml of standard PP stock solution (20 ppm) was transferred into the same volumetric flask. Then it was dissolved and made upto the volume with diluent and mixed well.

Preparation of placebo (other exicipients without AMB, PP, MP)

5 ml quantity of the placebo syrup was measured and transferred into a 50 ml volumetric flask. 30 ml of diluent was added and shaken for about 10 minutes, then diluted to the volume with diluent and mixed well (100 ppm).

Preparation of sample solution (Market product)

5 ml quantity of the syrup was measured and transferred into a 50 ml volumetric flask. 30 ml of diluent was added and shaken for about 10 minutes, then diluted to the volume with diluent and mixed well (100 ppm).

Table 1: Mobile Phase Composition for Gradient Programme

	Gradient Programme	
Time (min)	% Mobile phase A	% Mobile phase B
10	60	40
30	50	50
35	40	60
36	75	25
45	75	25

Table 2: Results for method precision

Sample No.	MP % content	PP % content	AMB % content
1	97.79	99.69	100.09
2	98.14	99.15	99.52
3	98.63	100.29	100.41
4	99.76	97.59	99.47
5	98.40	100.06	100.53
6	100.08	99.88	99.57
Average	98.8	99.44	99.93
%RSD	0.93	1.0	0.47

MP –Methyl paraben, PP - propyl paraben, % -Percentage

Table 3: Results for Linearity of Detector Response

Standard Concentration (ppm)						
Spike level	**MP**	**PP**	**AMB HCl**	**MP**	**PP**	**AMB HCl**
LOQ	0.03	0.06	0.08	2755	6588	2366
50 %	100	10	300	8314841	732975	8306036
75 %	150	14	450	12265022	1016367	12512814
100 %	200	20	600	16196878	1457357	16886395
125 %	250	24	750	19900857	1747815	21046005
150 %	300	30	900	23338916	2149861	24577451
Correlation Coefficient (r^2)				0.9994	0.9999	0.9997
Slope (m)				75368	71891	27608
Intercept (b)				92970	10224	84155
Bias for 100% response				0.57	0.70	0.50
LOD (ppm)				0.009	0.018	0.024
LOQ (ppm)				0.03	0.06	0.08

Ppm – parts per million, MP- methyl paraben, PP- propyl paraben, AMB HCL- Ambroxol hydrochloride, LOD- limit of detection, LOQ- Limit of quantification

Table 4: Results for Accuracy

Spike level	**Ppm added**			**Ppm recovered**			**Mean (% recovered)**		
	AMB	**MP**	**PP**	**AMB**	**MP**	**PP**	**AMB**	**MP**	**PP**
50 %	315.46	109.81	10	317.8	109.48	10.19	100.69	99.71	101.92
75 %	450.2	149.66	14	455.14	150.91	14.04	101.1	100.84	100.31
100 %	612.86	205.46	20	610.93	207.84	20.32	99.69	101.16	101.6
125 %	746.2	246.06	24	751.95	247.26	24.43	100.77	100.49	101.81
150 %	909.8	305.93	30	905.62	300.35	30.43	99.55	98.18	101.46

Ppm – parts per million

Table 5: Results for Forced Degradation

Stress condition	**%Net degradation**			**Peak Purity**						**Purity flag**
				AMB HCl		**MP**		**PP**		
	AMB	**MP**	**PP**	**PA**	**PT**	**PA**	**PT**	**PA**	**PT**	
2 N HCl	4.25	9.03	13.27	0.422	2.014	0.235	0.394	0.164	0.325	No
3 N Base	2.75	8.25	9.18	0.365	2.051	0.336	0.547	0.332	0.946	No
Peroxide	4.62	3.07	3.06	1.457	2.01	0.397	0.606	0.394	0.781	No
Sunlight	1.94	1.28	2.04	0.957	2.065	0.415	0.563	0.231	0.354	No
UV light	3.22	2.46	3.06	0.507	2.036	0.411	0.577	0.129	0.371	No
Thermal	0.31	0.29	0.32	0.506	2.10	0.493	0.677	0.103	0.329	No
Water	3.38	3.14	3.06	0.349	2.013	0.427	0.585	0.124	0.362	No

PA- purity angle, PT- purity threshold

Table 6: Results for Ruggedness (intermediate precision)

Parameters	**AMB**		**MP**		**PP**	
	I	**II**	**I**	**II**	**I**	**II**
Tailing factor	2.0	1.9	1.0	1.1	1.0	1.0
% RSD for peak area*	1.0	0.2	0.1	0.1	0.2	0.1
% RSD for assay*	0.47	0.25	0.93	0.25	1.0	0.78

(*n = 6) I = Using column I and system I, II = Using column II and system II

Table 7: Robustness data for AMB and its preservatives

Parameters	**AMB HCL RT**	**MP RT**	**PP RT**	**Acceptance Criteria**
Flow Rate				All the system suitability parameters should pass. The allowable variation in Flow rate of the method is from 0.8 ml/min to 1.2 ml/min.
0.8 mL/min	12.703	16.664	32.544	
1.0 mL/min	10.253	13.576	27.139	
1.2 mL/min	9.448	12.255	24.942	
Organic phase composition (%MeCN)				All the system suitability parameters should pass. The allowable variation is 90 % -110 %.
-10 %	12.835	16.044	34.122	
Actual	10.324	13.604	27.205	
+10 %	9.551	12.842	24.502	
Column temperature				
48°C	10.213	13.649	27.197	All the system suitability parameters should pass. The allowable variation in Column temperature is from 48°C to 52°C
50°C	10.24	13.604	27.205	
52°C	9.900	12.842	24.502	

RT- retention time, MeCN- Acetonitrile

Table 8: Bench Top Stability of Sample Preparation

Time in H	Similarity factor			Acceptance Criteria
	Ambroxol HCl	Methyl paraben	Propyl paraben	
Initial	NA	NA	NA	The similarity factor should be in the range of 0.95 to 1.05
01	1.00	0.99	0.99	
02	1.00	1.00	1.00	
07	1.00	1.00	1.01	

RESULTS AND DISCUSSION

Method development and optimization

Trifluoroacetic acid in the mobile phase was selected because, there were many possible ways of suppressing the interaction of residual silanols in the silica gel surface with basic analytes with frequently led to inferior separations due to the tailing of the peaks. The reduction of ionization of acidic SiOH sites by employing mobile phases of low pH or in contrast, the decrease of ionisation of the basic sample by increasing the pH of the mobile phase is the easiest method. Other approaches took advantage of the addition of silanol blockers e.g. triethylamine[21]. The optimization with mobile phase consisted of 0.1 % TFA: ACN (90:10 % v/v), flow rate 1.0 ml/min, Inertsil ODS C_{18} column and isocratic technique was done. Chromatogram showed unstable base line and resolution was found to be less. The optimization was continued by changing the volume of ACN in the in the mobile phase composition, gradient technique and same column it showed the split of AMB main peak, less resolution and less USP plate count for PP. The base line was unstable. So, the column Xterra RP_{18} was changed. As a result the peak shape was broad and tailing was observed for AMB peak. Then Hypersil BDS C_{18} column, volume of ACN and 0.1 % TFA should change (76: 24 % v/v) were used. Less USP plate count for pp and decrease the retention time were recorded. Inertsil ODS C_{18}, Xterra RP_{18}, Hypersil BDS C_{18} columns are having intermediate hydrophobicity and silanol activity. A stationary phase with lower hydrophobicity and silanol activity (Inertsil C_8, Symmetry RP_8) columns were chosen to reduce the secondary interactions leading to tailing in peaks and higher capacity factors for basic compounds. The role of stationary phases in reversed phase chromatography was a function of chain density and that the predominant driving force in the retention changed from an enthalpic to an entopic mechanism[22]. Finally the optimized conditions were Inertsil C_8 column, and mobile phase A consisted of 0.1 % TFA and mobile phase B consisted of ACN and mobile phase A (76: 24 % v/v). The optimized chromatogram was shown in Figure 4.

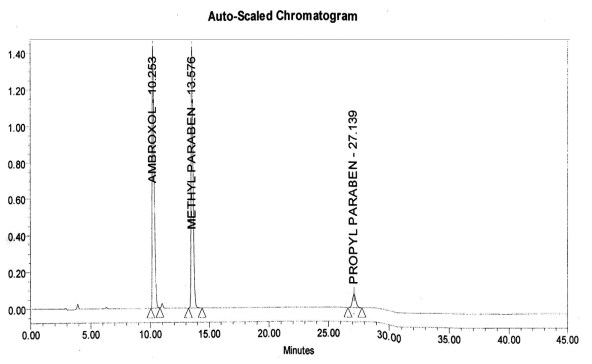

Figure 4: Optimized Chromatogram

Precision

The precision of the method was verified by repeatability and by intermediate precision. Method precision was evaluated by performing assay for six individual test preparations at the concentration of 100 ppm. % RSD of assay was calculated by using peak area and the chromatograms were recorded. The % RSD values were found to be less than 2 %. The percentage content of AMB HCL, MP and PP were found to be 98.8 %, 99.44 % and 99.93 % respectively within the limit (NLT 97.0 % and NMT 103.0 %). This indicated the method was precise. The results were shown in Table 2.

Linearity (plotting of calibration graph)

Study of linearity of detector response was done by injecting five solutions with spike levels of 50 %, 75 %, 100 %, 125 % and 150 % at the concentration ranging from 300 to 900 ppm for AMB, from 100 to 300 ppm for MP and from 10 to 30 ppm for PP. The peak area vs concentration data was treated by least square linear regression analysis. The correlation coefficients were found to be not less than 0.999. The results showed that an excellent correlation between the peak area and the concentration of the analyte and preservatives. Bias at 100 % responses was found to be not more than 2 %. The reports of analysis were shown in Table 3.

Detectability (LOQ and LOD)

The Limit of Detection (LOD) and Limit of Quantification (LOQ) of AMB HCl, MP and PP were determined based on signal to noise ratio method. For LOD, the concentrations of 0.009 ppm, 0.018 ppm, 0.024 ppm of AMB HCl, MP and PP were injected and the chromatogram was recorded. The signals to noise ratio values were calculated. For LOQ, the concentrations of 0.03 ppm, 0.06 ppm and 0.08 ppm of AMB HCl, MP and PP were injected and the chromatogram was recorded. The signals to noise ratio values were calculated. For LOD and LOQ S/N ratio values were found to be within the limit (LOD about 3.0 and LOQ about 10.0). The results were shown in Table 3.

Accuracy

The accuracy was confirmed by recovery studies. Recovery studies were performed by preparing six individual preparations at 50 % and 150 % level and triplicate preparation for remaining levels by adding AMB HCl, MP and PP in placebo. For each spike level to get the concentration of 50 %, 75 %, 100 %, 125 % and 150 % the concentrations of 300, 450, 600, 750, 900 ppm for AMB HCl, 100, 150, 200, 250, 300 ppm for MP and 10, 15, 20, 25, 30 ppm for PP were added. 20 µl of each concentration were injected into the chromatographic system and recorded the peak area. The average % recovery of AMB HCl, MP and PP was calculated and it was found to be 99.55 - 101.10 % for AMB, 98.18 - 101.16 % for MP and 100.31 - 104.62 % for PP respectively. The results were shown in Table 4. The average % recovery of AMB, MP and PP at each level was found to be within the limit (Not less than 97 % and not more than 103 %). This clearly indicated that the method was accurate and precise.

Specificity

(Forced degradation study)

Specificity study was conducted to demonstrate the effective separation of degradation product from AMB, MP and PP. Drug product and Placebo were exposed to following stress conditions to induce degradation separately. The solutions were treated with stress conditions of UV light for 7 days, heat (60˚ C for 2 hours), base (3N NaOH), acid (2N HCL), oxidation (30 % H_2O_2) and water (5 ml for 1 h at 60˚C) to evaluate the ability of the proposed method to separate AMB, MP and PP from its degradation product. For heat and light study period was about 10 days whereas, hydrolytic, acid, base and oxidation the study period was 10 hours. Peak purity was carried out for AMB, MP and PP peaks by using PDA detector in stress samples. Net % degradation was calculated by using peak area. It was found to be not more than 20 %. Purity angle must be less than the purity threshold. No purity flag was observed. Degradation product did not interfere with the AMB, MP and PP peaks. This study indicated that the method was specific and stability indicating. The report of analysis was shown in Table 5.

Ruggedness (intermediate precision)

The ruggedness of the method was demonstrated by conducting the precision study using different HPLC systems and different columns of same manufacturer at different days. Assay was performed for six individual sample preparations at the concentration of 100 ppm. 20 µl sample solutions were injected and the chromatograms were recorded. The system suitability parameters were calculated and % RSD for assay was calculated. The results were found within the limit. This clearly indicated that the method was precise. The report of analysis was shown in Table 6.

Robustness

To determine the robustness of the developed method, experimental conditions were deliberately altered and the resolution between AMB, MP and PP and tailing factor for AMB and its preservatives were recorded. The flow rate of the mobile phase was 1.0 ml/min. To study the effect of flow rate on the resolution, flow rate was changed by 0.2 units from 0.8 to 1.2 ml/min. The effect of column temperature on resolution was studied at 48˚C and 52˚C instead of 50˚C. The effect of the percent acetonitrile strength (mobile phase B) on resolution was studied by varying acetonitrile from -10 to +10 %. The resolution between AMB and its preservatives was greater than 2.0 min and tailing factor for AMB, MP and PP was less than 2.0. The % RSD for peak area was within 2.0 %. The results were shown in Table 7.

Solution stability

Solution stability studies were carried out by keeping the sample preparation on bench top at room temperature for 24 hours and the mobile phase was prepared as per the test method and kept it on bench top in well closed conditions for 7 days. System suitability parameters were evaluated by injecting freshly prepared standard each time as described in the test method by using stored mobile phase at initial, after day 1, after day 2 and day 7. The system suitability parameters were calculated and the mobile phase stability was observed on bench top. The sample solutions were injected at initial time, 1 h, 2 h and 7 h. The similarity factor was calculated and the sample solution stability was observed. The results were shown in Table 8. The system suitability parameters values and similarity factor values were obtained within the limit. No significant changes were observed in sample preparation and mobile phase during solution stability experiments. The solution stability experiments data confirms that the sample solution was stable for 7 h and mobile phase was stable for 7 days.

CONCLUSION

A simple gradient reversed phase HPLC method was found to be accurate, precise, linear across the analytical range and robust. The method was specific for the determination of AMB, MP and PP in an oral liquid formulation. All the parameters for AMB, MP and PP meet the criteria of the ICH guidelines for method validation. There were no interference peaks in the chromatograms, therefore no additional extractions or separations are required. The method is rapid and sensitive enough to be used for oral dosage forms.

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Source of support: Nil, Conflict of interest: None Declared

QUICK RESPONSE CODE	ISSN (Online) : 2277 –4572
	Website http://www.jpsionline.com

How to cite this article:
Sudha T, Manthena Keerthi, Ravikumar V.R, Ganesan V. HPLC method for the determination of ambroxol HCl in the presence of antimicrobial preservatives in oral liquid formulation. J Pharm Sci Innov. 2015;4(2):134-139 http://dx.doi.org/10.7897/2277-4572.04230