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High Performance Liquid Chromatographic Method for the Determination of Ambroxol Hydrochloride in Presence of Antimicrobial Preservatives in Oral Liquid Formulation

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Abstract

A simple gradient reversed phase high performance liquid chromatographic method was developed for the determination of ambroxol hydrochloride in presence of antimicrobial preservatives in oral liquid formulation. The chromatographic separation was achieved by an Inertsil C_8 (250 X 4.6 mm, 5µ particle size) column using gradient technique. The eluents were detected at 245 nm with photodiode array 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. Ambroxol hydrochloride and microbial preservatives were eluted at a flow rate of 1.0 ml/min. The method validated according to the International Conference of Harmonization (ICH) guidelines. The calibration curves were linear over the ($r^2 > 0.99$) concentrations 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 limit of detection was found to be 0.024 ppm for ambroxol hydrochloride, 0.018 ppm for propyl paraben and 0.009 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 101.46% 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.

Key words: Ambroxol hydrochloride, Methylparaben, Propylparaben, Method validation, Forced degradation

Introduction

Ambroxol hydrochloride (AMB) chemically trans-4-(2-Amino-3,5-dibrombenzylamino) cyclohexanol hydrochloride (Figure 1a) is a semi-synthetic derivative of vasicine obtained from Indian shrub *Adhatoda vasica* (Family- Acanthaceae). It is a metabolic product of bromhexine. It is used as broncho secretolytic and an expectorant drug (Budavari 2001). It simulates the transportation of the viscous secretions in the respiratory organs and reduces the stand stillness of the secretions. Methylparaben (MP) chemically known as methyl 4-hydroxybenzoate (Figure 1b), and propylparaben (PP) chemically known as propyl 4-hydroxybenzoate (Figure 1c) 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 (The Pharmaceutical Codex 1994). Several different methods have been reported for individual determination of AMB in pharmaceutical preparations by spectrophotometry (Kuchekar *et al.,* 2003; Reddy *et al.,* 1998; Zafer *et al*., 2003; Gunawan and Ratna, 1993).

Determination of AMB in biological fluids by capillary electrophoresis with fluorescence detection (Tomas *et al.,* 2000), gas chromatography with electron

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capture detection (Colombo 1990), capillary gas liquid chromatography (Schmid 1987), HPLC with amperometric detection (Flores *et al.,* 1989) and LC-MS/MS (Hohyun *et al.,* 2003) methods have been reported. Several methods have also reported for the determination of AMB like as AMB with bromhexine by capillary isotachophoresis (Polasek *et al.,* 2001), simultaneous determination of AMB and cetirizine in their tablet formulation by absorbance ratio method (Kokil *et al.,* 2008), determination of AMB and doxycycline by novel reversed phase sequential injection chromatography (Araujo *et al.,* 2005**)**, AMB in combination with BA (benzoic acid) in syrup as pharmaceutical dosage form for stress test as stability evaluation by HPLC (Maarit and Coral, 2001), AMB with MP and BA in pharmaceutical preparations based on sequential injection technique coupled with monolithic column (Dalibor *et al.,* 2006), AMB with different preservatives in pharmaceutical formulations by HPLC (John *et al.,* 2000). However, to the best of our knowledge, HPLC method for the determination of AMB in oral liquid preparations, co-elution of AMB with only parabens or other excipients were not reported in the literature. The present paper describes the determination of AMB in oral liquid preparations containing 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.

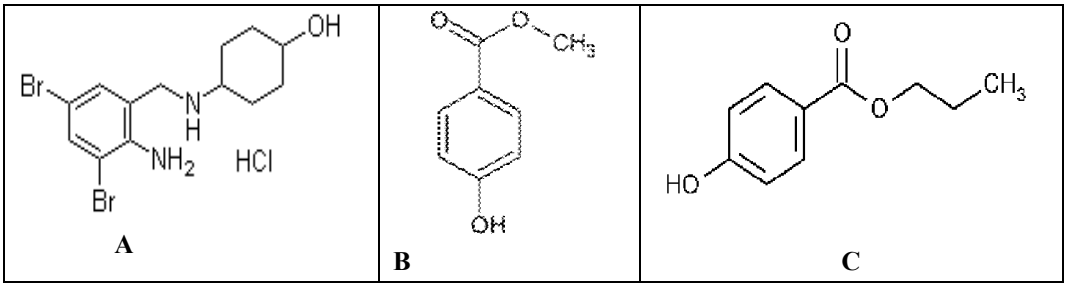

Figure 1. Chemical structures of ambroxol hydrochloride (A); methylparaben (B) and propylparaben (C).

Materials and Methods

Materials and reagents: Ambroxol hydrochloride, marketed formulation, placebo syrup, propyl and methyl parabens were kindly donated by Dr. Reddy's Laboratories Ltd., 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 Ltd., 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, Germany). Thermal stability studies were performed in a dry air oven (Cintex, Mumbai, India).

Preparation of standard solution

Preparation of standard stock solution for PP: 40 mg of PP 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 MP and AMB: 40 mg of MP working standard (200 ppm) and 120 mg of AMB (600 ppm) were accurately weighed and transferred into a 200 ml volumetric flask. 100 ml of diluent was added and sonicated to dissolve the MP and drug. 10 ml of standard PP stock solution (20 ppm) was transferred into the same volumetric flask. Then it was mixed properly 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 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).

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, 5µm (250 mm X 4.6 mm) column. Solvent A consisted of 0.1 % trifluoroacetic acid (TFA) 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 at the ratio of 50:50 (v/v) was used as a diluent. 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.

Table 1. Mobile phase composition for gradient program.

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

Results and Discussion

Method development and optimization: TFA 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 ionization of the basic sample by increasing the pH of the mobile phase is the easiest methods. Other approaches took advantages of the addition of silanol blockers e.g. triethylamine (Sykora *et al.,* 1997). The optimized chromatographic conditions were developed with Inertsil C_8 column, and mobile phase A of 0.1% TFA and mobile phase B consisted of acetonitrile and mobile phase a (76:24% v/v). The optimized chromatogram was shown in Figure 2.

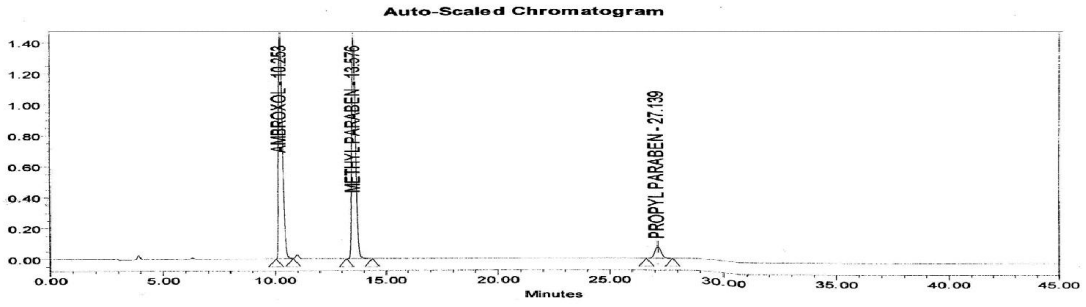

Figure 2. Optimized chromatogram.

Method validation: The method was validated as per ICH guidelines (ICH Q1A (R2) 2005; ICH Q2A (R1) 2005).

System precision: Precision is the degree of reproducibility or repeatability of the analytical method under normal operating condition. The precision of the method was verified by repeatability and intermediate precision. Standard solutions were prepared by using AMB (600 ppm), MP (200 ppm) and PP (20 ppm) as per test method and injected five times into the HPLC system. % RSD of assay was calculated by using peak area of the chromatograms. The %RSD values were found to be less than 2%. The % RSD for peak areas from five replicate injections of AMB, MP and PP were found to be 0.1, 0.1 and 0.2, respectively. The %RSD value of precision was found lower than that obtained by the previous method

(Dalibor 2006; John 2000). Therefore, the developed method indicated precise. The results are shown in Table 2.

Linearity (plotting of calibration graph): The linearity of an analytical method is its ability to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to the concentration of analyte in samples within a given range. The linearity of the method was observed with in the expected concentration range demonstrating its suitability for analysis. 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 concentration of linearity range was lower than the previously published method (Maarit and Coral, 2001). 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.

Table 2. Reports for validation parameters.

Validation parameters	Ambroxol hydrochloride	Methylparaben	Propylparaben	Reference value
Linearity study				
Range (ppm)	300-900	100-300	10-30	50-150 % for
Correlation coefficient (r^2)	0.9997	0.9994	0.9998	AMB , 0.15-
Slope (m)	27608	75368	71891	0.45mg/ml
Intercept (c)	84155	92970	10224	
Limit of detection (ppm)	0.024	0.009	0.018	
Limit of quantification (ppm)	0.08	0.03	0.06	
Precision study				
System precision (%RSD)	0.1	0.1	0.2	
Ruggedness study				
Column (I) & system (I)				0.44 for AMB
Tailing factor	2.0	1.0	1.0	
Peak area (%RSD)	1.0	0.1	0.2	
Assay (% RSD)	0.47	0.93	1.0	
Column (II) & system (II)				
Tailing factor	1.9	1.1	1.0	
Peak area (% RSD)	0.2	0.1	0.1	
Assay (% RSD)	0.25	0.25	0.78	

%RSD = Percentage relative standard deviation.

Detectability (LOQ and LOD): The detection limit or LOD is the lowest amount of an analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions. It may be expressed as a concentration that gives a signal to noise ratio of 3:1. Quantitation limit or LOQ is the lowest of analyte in a sample that can be determined with acceptable precision and accuracy under the experimental conditions. A signal to noise of 10:1 can be taken as LOQ of the method. The Limit of Detection (LOD) and Limit of Quantification (LOQ) of AMB, 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, MP and PP were injected and the chromatograms were recorded. For LOQ, the concentrations of 0.03 ppm, 0.06 ppm and 0.08 ppm of AMB, MP and PP were injected and the chromatograms were recorded.

Accuracy: The accuracy of an analytical method is the closeness of test results obtained by the method to true value. 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, MP and PP in placebo. For each spike level to get the concentration of 50 - 150% the concentrations of 300-900 ppm for AMB, 100 - 300 ppm for MP and 10 - 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, MP and PP were calculated and it was found to be 99.55 - 101.10% for AMB, 98.18 - 101.16% for MP and 100.31 - 101.46%

for PP (Chitra *et al.,* 2012). The results are shown in Table 3. The average % recovery of AMB, MP and PP at each level was found to be within the limit. 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 (60ºC for 2 hrs), base (3N NaOH), acid (2N HCl), oxidation (30% H_2O_2) and water (5ml for 1 hr 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, the study period was about 10 days whereas for hydrolytic, acid, base and oxidation, the study period was 10 hrs. 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 and 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 showed that the method was specific and stability indicating. The report of analysis is shown in Table 4.

Table 3. 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	101.42
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	101.46
125%	746.2	246.06	24	751.95	247.26	24.43	100.77	100.49	101.11
150%	909.8	305.93	30	905.62	300.35	30.43	99.55	98.18	101.46

ppm = parts per million

Table 4. Results for forced degradation.

Stress condition	% Net degradation			Peak purity						Purity flag
	AMB	MP	PP	AMB		MP		PP		
				PA	PT	PA	PT	PA	PT	
2N HCl	4.25	9.03	13.27	0.422	2.014	0.235	0.394	0.164	0.325	No
3N 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

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 AMB (600 ppm), MP (200 ppm) and PP (20 ppm). The injection volume for each time was kept at 20 µl and the chromatograms were recorded. The system suitability parameters were calculated and expressed as % RSD. The results were found within the limit. This clearly indicated that the method was precise. The results are shown in table 2.

Robustness: To determine the robustness of the developed method, experimental conditions were deliberately altered and the resolutions as well as the tailing factors of AMB, MP and PP were evaluated. To

study the effect of flow rate on the resolution, flow rate was changed by ± 0.2 units from 1 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 (mobile phase B) on resolution was studied by varying acetonitrile by ± 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 are shown in Table 5.

Table 5. 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.
0.8ml/min	12.703	16.664	32.544	The allowable variation in flow rate of the method is from 0.8 ml/min to 1.2 ml/min.
1.0ml/min	10.253	13.576	27.139	
1.2ml/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				All the system suitability parameters should pass.
48°C	10.213	13.649	27.197	The allowable variation in column temperature is from 48°C to 52°C
50°C	10.324	13.604	27.205	
52°C	9.900	12.84	24.502	

RT- retention time, MeCN- Acetonitrile

Table 6. Bench top stability of sample preparation.

Time in hr	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	

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 the 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 hr, 2 hrs and 7 hrs. The similarity factor was calculated and the sample solution stability was observed. The results are shown in Table 6. 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 hrs and mobile phase was stable for 7 days.

Conclusion

A simple, precise, accurate and gradient RP-HPLC method was developed for the analysis of AMB and anti-microbial preservatives in oral liquid pharmaceutical formulations. The correlation coefficient r^2 of AMB, MP and PP were found to be 0.9997, 0.9994 and 0.9998,

respectively. Nearly 100 % recovery showed that the method was free from the interference of the excipients used in the formulation. The % RSD within limits indicated that the method was precise. Since all the acceptance criteria of the parameters selected for validation were found to be satisfied. Therefore, the proposed method was rapid, accurate, simple, precise, and robust enough and could be effective for routine analysis of AMB, MP and PP present in oral liquid dosage form.

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