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HPLC Method for Simultaneous Determination of Ambroxol, Salbutamol and Fexofenadine in Their Bulk and Dosage Forms

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ABSTRACT

An isocratic HPLC method has been developed for determination of ambroxol, salbutamol, and fexofenadine in their pure and tablet forms. Separation was carried out at room temperature on an Equisil BDS C18 (5 um, 4.60 mm x 150 mm) column using a mobile phase of acetonitrile and 0.1% ortho-phosphoric acid (20:80, v/v). The flow rate was 1 mL/min, maximum absorption was measured at 220 nm and linearity was in the range of 1-20 µg/mL for all drugs. The retention times of ambroxol, salbutamol, and fexofenadine were reported to be 2.35, 3.72 and 5.35 minutes respectively, indicating a very short analysis time rather than other reported methods. Also, limits of detection were reported to be 0.13, 0.08 and 0.11 µg/mL for ambroxol, salbutamol, and fexofenadine respectively, showing a high degree of the method sensitivity. The proposed method was validated in terms of linearity, accuracy, precision and robustness according to ICH guidelines and results were compared statistically with reference methods in respect of precision and accuracy.

INTRODUCTION

Salbutamol (SAL), 4-[2-(tert-butylamino)-1-hydroxyethyl]-2-(hydroxymethyl) phenol, (Figure 1) is a short-acting, selective beta2-adrenergic receptor agonist used in the treatment of asthma and chronic Obstructive Pulmonary Disorder (COPD) [1]. It is 29 times more selective for beta2 than beta1 receptors giving it higher specificity for pulmonary beta receptors versus beta1-adrenergic receptors located in the heart [2]. SAL is formulated as a racemic mixture of the R- and S-isomers. The R-isomer has 150 times greater affinity for the beta2-receptor than the S-isomer while the latter has been associated with toxicity [3]. This lead to the development of levalbuterol, the single R-isomer of SAL. However, the high cost of levalbuterol compared to SAL has deterred wide-spread use of this enantiomerically pure version of the drug [4]. SAL is generally used for acute episodes of bronchospasm caused by bronchial asthma, chronic bronchitis [5], for the prevention of exercise induced bronchospasm and other chronic bronchopulmonary disorders such as COPD [6]. It is also used prophylactically for exercise-induced asthma [7]. Some analytical methods were reported for the determination of SAL in its pharmaceutical or biological samples such as liquid chromatography [8-14], and spectrophotometry [15-17].

Ambroxol (AMB), (1r,4r)-4-{[(2-amino-3,5-dibromophenyl)methyl]amino}cyclohexan-1-ol, (Figure 1) is a mucolytic agent used in the treatment of respiratory diseases associated with viscid or excessive mucus secretion [18]. It is a mucoactive drug with several properties including secretolytic and secretomotoric actions that restore the

physiological clearance mechanisms of the respiratory tract which play an important role in the body's natural defense mechanisms [18]. It stimulates synthesis and release of surfactants by type II pneumocytes [19]. Some analytical methods were published for the determination of AMB either by liquid chromatography [20-25], or spectrophotometrically [26,27].

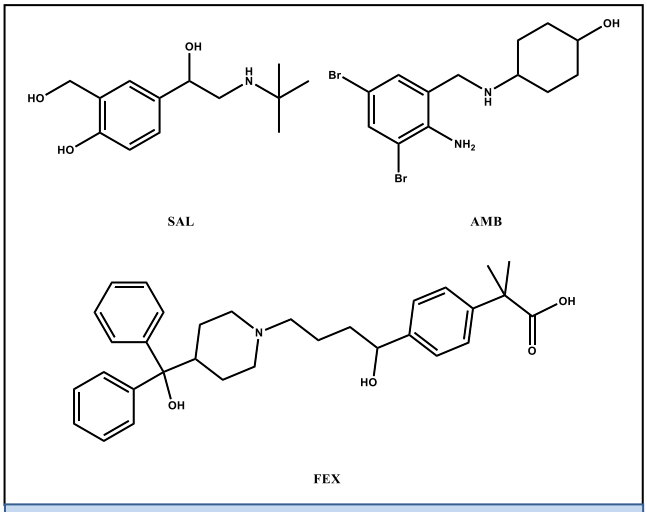

Figure 1: Chemical structures of Salbutamol (SAL), Ambroxol (AMB) and Fexofenadine (FEX).

Fexofenadine (FEX), 2-(4-{1-hydroxy-4-[4-(hydroxydiphenylmethyl) piperidin-1-yl]butyl}phenyl)-2-methylpropanoic acid, (Figure 1) is an antihistamine drug used in the treatment of hay fever and similar allergy symptoms. It was developed as a successor and alternative to terfenadine [28]. FEX, like other second and third-generation antihistamines, does not readily pass through the blood-brain barrier, and so causes less drowsiness than first-generation histamine-receptor antagonists [29]. Some analytical methods were published for the determination of FEX by liquid chromatography [30-34], and spectrophotometry [35-37].

To the best of our knowledge, there is no method that has been reported for the simultaneous chromatographic separation of the three drugs despite their synergistic action. As such, the present work introduces a simple, rapid, reproducible and sensitive chromatographic method that has been established and validated for the determination of the antiallergic mixture in their pure forms and in their tablet dosage form according to ICH guidelines [38].

MATERIALS AND METHODS

Instrumentation

High Performance Liquid Chromatography (HPLC) apparatus is equipped with Surveyor quaternary pump with Intel vacuum degasser (Agelint 1100), a Surveyor autosampler plus (Thermo Scientific Co., USA), Equisil BDS C18 (5 um, 4.60 mm x 150 mm) column (Thermo Scientific Co. USA), Autosampler vials 1.8 mL screw cap (Thermo Scientific, USA), and Surveyor photodiode array detector (PDA) (Thermo Scientific Co. USA). A computer with a software chromo quest 5 (Surveyor Thermo Scientific Co. USA), has been used for data collection and analysis,. Consort P400® digital pH-meter was used for pH adjustment.

Chemicals and reagents

All solvents and reagents were of HPLC analytical grade. Acetonitrile HPLC grade was supplied by Fischer scientific (Loughborough, UK), ortho-phosphoric acid was purchase from Merck (Darmstadt, Germany) and water used in all the experiments was obtained from Milli-RO and Milli-Q systems (Millipore, Bedford, MA). Standard powders of SAL, FEX and were kindly supplied by EIPICO (Egypt) while AMB was provided by Adwia company (EGYPT).

Chromatographic conditions

HPLC was connected with 5 um Equisil BDS C18 (4.60 mm x 150 mm) column as a stationery phase. A mixture of acetonitrile and 0.1% ortho-phosphoric acid in a ratio of 20:80 v/v was prepared and used as an isocratic mobile phase. The mobile phase was pumped at a flow rate of 1mL/min. The injection volume was 10 µL and the column was maintained at ambient temperature while the eluent was monitored at 220 nm. All chromatographic conditions are illustrated in (Table 1).

Table 1: Chromatographic Conditions for the proposed method.

Parameters	Conditions
Column	Equisil BDS C_{18} (5 um, 4.60 mm x 150 mm)
Mobile phase	Filtered and degassed isocratic acetonitrile : 0.1% ortho-phosphoric acid in water (20:80)
UV detection, nm	220
Flow rate, mL/min	1
Injected volume, µl	10
Temperature	Ambient (25±5°C)

Preparation of standard stock solution and construction of calibration curves

Standard stock solution of SAL, AMB, and FEX (100 µg/mL) were prepared by dissolving 10 mg of each pure drug in 100 mL water. Then, Standard solution was diluted by methanol to get final concentrations of 1, 5, 10, 15, and 20 µg/mL for all drugs for construction of calibration plots. The mixture was injected in triplicate and chromatographed under the previously mentioned conditions. A linear relationship was obtained when average drug standard peak areas were plotted against the corresponding concentrations for each drug and regression equations were computed.

Pharmaceutical preparations

10 Tablets (30 mg AMB, 4 mg SAL and 120 mg FEX) were weighed and finely powdered. An accurately weighed portion from the powdered tablets equivalent to the average weight of one Tablet (250 mg) was transferred into a 100 mL volumetric flask. 80 mL of water were added and sonicated for 20 minutes then the volume was complete with diluent to 100 mL and filtered. Further dilution was performed to obtain the required concentration range of the drug mixture.

RESULTS AND DISCUSSION

Optimization of chromatographic condition

Several trials were carried out to obtain the optimized chromatographic condition for simultaneous determination of SAL, AMB, and FEX. First, chromatographic detection was performed at 220, 215, and 210 nm using a PDA detector and the optimal wavelength was set at 220 nm. Second trials were carried out by changing mobile phase composition, as shown in Figure 2 (A-D), to reach the optimum separation with good resolution and the mobile phase of acetonitrile : 0.1% ortho-phosphoric acid with ratio (20 : 80) was chosen as the optimum one based of faster separation and good peak resolution. Last trials were carried out to show effect of different flow rates and optimal separation was achieved at a flow rate of 1 mL/min. Under these conditions, AMB, SAL, and FEX in pure form were separated and eluted at 2.35, 3.72 and 5.35 minutes respectively as illustrated in Figure 2(C) A and in dosage form as illustrated in Figure 3. However, the optimum mobile phase showed symmetrical peaks ($0.97 < T < 1.03$), capacity factor ($1 < k < 10$), resolution > 2 and theoretical plates > 2000 which are in agreement with the CDER values recommendation [39]. Table 2 shows all system suitability parameters of the proposed RP-HPLC method for simultaneous determination of the three drugs in pure form.

Table 2: System suitability parameters for Ambroxol (AMB), Salbutamol (SAL), and Fexofenadine (FEX) in their pure form.

Parameters	AMB	SAL	FEX	Reference values [39]
Retention time, tr	2.35	3.72	5.35	
Capacity factor, k'	1.14	2.38	3.86	Accepted k' value (1-10)
Peak asymmetry (Tailing factor, T)	1.03	0.97	0.99	Accepted T value ≤ 2
Therotical plates, N	2209	5535	11449	Accepted N value > 2000
Resolution, Rs	---	6.85	8.15	Accepted value > 2
Selectivity (Separation factor, α)	---	2.09	1.62	

Method validation

The proposed method was validated according to ICH guidelines [38] in terms of specificity, linearity precision, accuracy, robustness, limit of detection and limit of quantification.

Specificity: Specificity, is the ability of an analytical method to distinguish the analyte from other chemicals in the sample. The specificity of the method was assessed by deliberately adding impurities into a sample containing the analyte and testing how well the method can identify the analyte. It was found that there was no interference due to excipients found in tablet formulation as seen in (Figure 3).

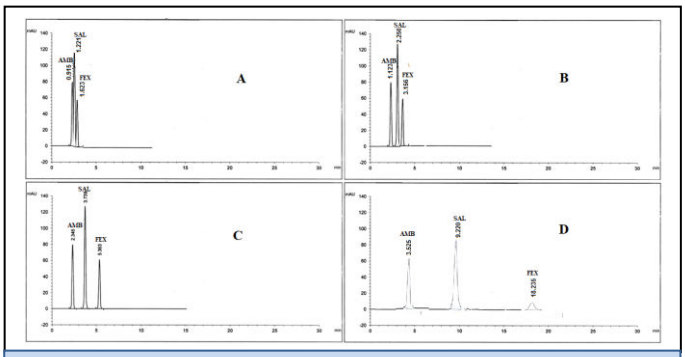

Figure 2: Typical HPLC chromatograms obtained from pure AMB, SAL, and FEX using 5um Equisil BDS C18 (4.60 mm × 150 mm) column using different mobile phase compositions of ACN: 0.1% ortho-Phosphoric acid as follow (A) 40:60, (B) 30:70, (C) 20:80 and (D) 5:95.Other chromatographic conditions are stated in Table 1.

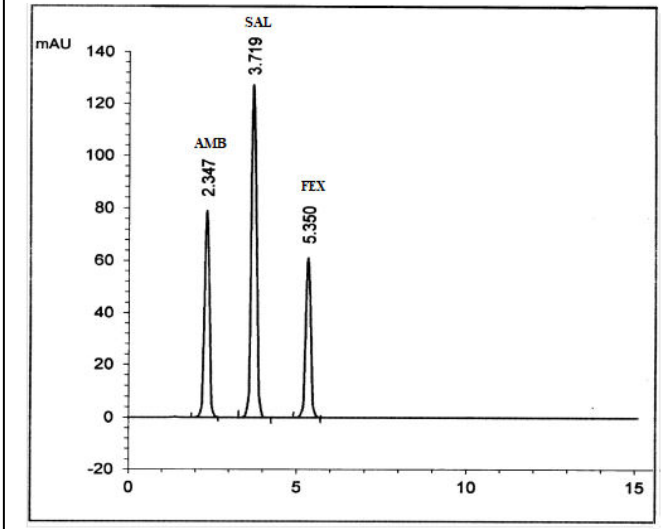

Figure 3: Typical HPLC chromatograms obtained from mixture of AMB, SAL, and FEX in their pharmaceutical tablet formulation under optimized chromatographic conditions in Table 1.

Table 3: Results of analysis for Ambroxol (AMB), Salbutamol (SAL), and Fexofenadine (FEX) in pure form using the proposed method.

AMB				SAL				FEX			
Taken µg/mL	Area under curve	Found µg/mL	Recovery %	Taken µg/mL	Area under curve	Found µg/mL	Recovery %	Taken µg/mL	Area under curve	Found µg/mL	Recovery %
1	42.24	0.98	98.47	1	55.19	1.003	100.32	1	31.76	1.01	101.17
5	212.91	5.14	102.86	5	281.59	5.103	102.06	5	162.00	5.07	101.47
10	406.41	9.86	98.58	10	540.32	9.79	97.89	10	315.29	9.85	98.54
15	618.77	15.03	100.21	15	833.65	15.10	100.67	15	481.22	15.03	100.19
20	823.21	20.01	100.07	20	1104.4	20.004	100.02	20	641.70	20.032	100.16
Mean			100.04				100.19				100.30
±SD			1.77				1.51				1.15
±RSD			1.77				1.50				1.14
±SE			2.05				1.70				1.10
Variance			3.15				2.27				1.31
LOD.			0.13				0.08				0.11
LOQ.			0.43				0.27				0.36

Table 4: Results of Intra-day and inter-day precision of the three drugs.

Drug	conc. µg/mL	Intra-day		Inter-day	
		mean± SD	RSD	mean± SD	RSD
AMB	5	100.60 ± 1.80	1.80	100.40 ± 1.70	1.70
	10	101.00 ± 0.97	0.97	101.00 ± 1.47	1.45
	15	100.2 0 ± 1.20	1.20	98.30 ± 0.59	0.59
SAL	5	100.40 ± 1.43	1.42	101.30 ± 1.39	1.37
	10	99.60 ± 0.90	0.90	99.90 ± 1.50	1.50
	15	100.40 ± 1.40	1.40	101.00 ± 1.19	1.18
	5	100.80 ± 1.80	1.80	100.60 ± 1.70	1.70
FEX	10	102.00 ± 0.97	0.97	101.00 ± 1.47	1.45
	15	100.10 ± 1.20	1.20	98.30 ± 0.59	0.59

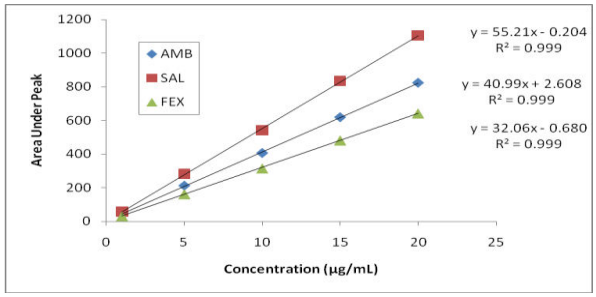

Figure 4: Calibration curves of AMB, SAL and FEX using proposed HPLC method.

Table 5: Results of accuracy study (recovery).

DRUG		AMB		SAL		FEX	
		Mean ± SD	RSD	Mean ± SD	RSD	Mean ± SD	RSD
conc. µg/mL	5	100.20 ± 0.10	0.10	99.00 ± 0.37	0.37	100.30 ± 0.37	0.37
	10	100.40 ± 0.16	0.17	99.70 ± 0.29	0.29	100.70 ± 0.29	0.29
	15	98.90 ± 0.95	0.95	101.76 ± 0.10	0.10	100.76 ± 0.10	0.10

Table 6: Results of robustness.

Drug	AMB Mean ± SD	SAL Mean ± SD	FEX Mean ± SD
Flow rate 1.05	100.20 ± 1.40	100.90 ± 1.10	99.95 ± 1.10
Flow rate 0.95	100.30 ± 1.49	100.30 ± 1.17	99.90 ± 1.17
Mobile phase 19:81	100.60 ± 1.35	100 .50 ± 0.05	101.20 ± 1.00
Mobile phase 21:79	100.30 ± 1.30	100.40 ± 1.00	100.2 ± 1.20

Linearity: Five different concentrations of the drug mixture were specified for linearity studies in the range of 1-20 µg/mL for all drugs as seen in (Table 3). A linear relationship was established by plotting concentrations against corresponding peak areas. The correlation coefficient was around 0.999 indicating good linearity as shown in (Figure 4). Also, the regression equations were found to be y=40.99x +2.6082, y= 55.219x - 0.2045, and y=32.066x -0.6807, for AMB, SAL and FEX respectively.

Limits of detection and quantification: Limit of Detection (LOD) of an analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. Limit of Quantification (LOQ) is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. LOD = 3.3 S/K and LOQ = 10 S/K, were used for the values calculation where S is the standard deviation of three replicate determination values under the same conditions and K is the slope of calibration graph. LODs were reported to be 0.13, 0.08 and 0.11 µg/mL, while LOQs were calculated to be 0.43, 0.27, and 0.36 µg/mL for AMB, SAL and FEX respectively (Table 3). These results show that the proposed method is highly sensitive and applicablee not only for pharmaceutical analysis but also for pharmacokinetic studies.

Precision: The precision of the method was calculated in terms of repeatability and intermediate precision (intra-day and inter-day precision), the results were represented in (Table 4) by calculating Standard Deviation (SD) of five replicate determinations using the same solution containing pure drug during the same day and five consecutive days. The SD values (0.90 to 1.80) for intra-day and those (0.59-1.70) of inter-day precision were in the acceptable range and showed that the proposed method has an adequate precision in respect of the simultaneous determination of the 3 cited drugs in their pharmaceutical formulation.

Accuracy and recovery: Accuracy was assessed using 9 determinations over 3 concentration levels of 5, 10, and 15 µg/mL covering the specified ranges. The results showed excellent recoveries with lower SD values as seen in (Table 5).

Robustness: Robustness of an analytical procedure is a measure of its capacity to remain unaffected by small variations in method parameters. In the proposed method, a small variation in the flow rate and mobile phase composition showed a negligible effect on the on the results as revealed by small SD values (SD ≤ 1.49) for all applied changes (Table 6).

Application on pharmaceutical preparation

The proposed method was successfully applied on pharmaceutical preparation containing AMB, SAL and FEX. Results obtained were established in (Table 7), showing a high degree of accuracy and precision where excipients and

impurities did not show interference on the selected values. Also, results obtained were compared to those obtained by reference methods [11,22,34] where Student's t-test and F-test were performed for comparison. Results shown in (Table 8) indicated that calculated t and F values were less than tabulated ones for the 3 drugs which in turn indicate that there is no significant difference between proposed method and reference ones relative to precision and accuracy.

Table 7: Results of analysis of Ambroxol (AMB), Salbutamol (SAL), and Fexofenadine (FEX) in pharmaceutical formulation®.

AMB				SAL				FEX			
Taken µg/mL	Area under curve	Found µg/mL	Recovery %	Taken µg/mL	Area under curve	Found µg/mL	Recovery %	Taken µg/mL	Area under curve	Found µg/mL	Recovery %
1	43.25	1.01	100.93	**1**	55.25	1.004	100.43	**1**	31.25	0.99	99.58
5	210.25	5.08	101.57	**5**	282.35	5.12	102.34	**5**	160.23	5.02	100.36
10	407.25	9.88	98.78	**10**	550.25	9.97	99.69	**10**	317.25	9.91	99.15
15	619.25	15.04	100.29	**15**	834.25	15.11	100.74	**15**	481.22	15.03	100.19
20	825.25	20.06	100.32	**20**	1105.12	20.02	100.09	**20**	641.70	20.03	100.16
Mean			100.38				100.66				99.89
±SD			1.03				1.02				0.50
±RSD			1.02				1.01				0.51
±SE			1.10				1.10				0.40
Variance			1.07				1.04				0.26

Table 8: Statistical analysis of results obtained by the proposed HPLC method applied on pharmaceutical formulation compared with reference methods.

DRUG	Recovery ± SD				Reference method number	Student t- values	F- Values
	Proposed Method	N	Reference Method	N			
AMB	100.40 ± 0.46	5	99.43 ± 0.24	3	[22]	1.47 (1.94)[a]	6.19 (19.25)[b]
SAL	100.70 ± 0.45	5	99.52 ± 0.24	3	[11]	1.80 (1.94)[a]	5.93 (19.25)[b]
FEX	99.89 ± 0.23	5	100.00 ± 0.10	3	[34]	0.41 (1.94)[a]	8.41 (19.25)[b]

[a] and [b] are the Theoretical Student t-values and F-ratios at p=0.05.

CONCLUSION

A simple, precise, accurate, valid, robust, highly sensitive and reliable HPLC method was established for determination of ambroxol, salbutamol, and fexofenadine in bulk and pharmaceutical preparation. In the proposed method the chromatographic resolution was achieved within less than 6 minutes for the three drugs. Linearity was observed over a concentration range of 1-20 µg/mL for all drugs. The method has been successfully applied for the analysis of tablet formulation in respect of quality control in addition to performing statistical comparison with reference methods showing no significant differences.

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