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Reverse phase-UPLC method of analysis for simultaneous estimation of Levosalbutamol sulphate, Guaiphenesin and Ambroxol hydrochloride in pharmaceutical cough, cold liquid dosage forms

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ABSTRACT

The three most drug combinations for cough, cold are widely used worldwide now a day. The purpose of the study was to build up an innovative RP-UPLC technique for simultaneous estimation of Levosalbutamol Sulphate (LEV), Guaiphenesin (GUA) and Ambroxol Hydrochloride (AMB) in liquid dosage forms. Chromatography was carried out on UHPLC (WATERS)_SYMMETRY® C18 4.6mm x 1000mm, 3.5µm, (Agilent - Zorbax Eclipse Plus C18 – Rapid Resolution) with an isocratic mobile phase with pH 3.0 composed of buffer, methanol and Acetonitrile (60:20:20) with a flow rate of 0.8mL/min. The detection was carried out with column temperature at 25°C using a UV detector at 276nm. Validation parameters like linearity, specificity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), system suitability, Solutions stability and robustness were considered as affirmed in the ICH guidelines. Retention times for LEV, GUA & AMB were 1.07 min, 1.99 min & 3.55 min respectively. The assay of syrups with the relative standard deviation found to be less than 2%. The parameters values were found, and the method was found to be satisfactory. This validated UHPLC method is cost-effective, receptive and precise than other chromatographic methods.

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INTRODUCTION

The very common diseases in human are cold, and cough and are normally treated with multiple dosage forms. Cough is a protective impulse of the human body which removes the toxic materials from the respiratory tract and is a common cause of angiotensin-converting enzyme inhibitors (Rang HP *et al.,* 2009). The above-mentioned combination is for clinical relief of cough related to bronchial asthma, emphysema, bronchitis and the other Broncho pulmonary disorders where mucous plugging, bronchospasm and expectoration problems co-exist.

Levosalbutamol sulphate (LEV) (Fig.1) also known as Levalbuterol, a short-acting β2 adrenergic receptor agonist and its molecular formula C13H21NO3, Molecular Wt. 239.311 g/mol used in the treatment of Bronchial asthma (Maryadele.J, 2006), Chronic Bronchitis and Chronic obstructive pulmonary disease (COPD)(Albuterol.html.2011). Activation of adenylate cyclase and an increase in the intracellular concentration of 3', 5'-cyclic adenosine monophosphate (cyclic AMP) is caused by activation of β2 adrenergic receptors on airway smooth muscle. Inhibiting the phosphorylation of myosin and lowering of intracellular ionic calcium concentrations increases in cyclic AMP associated

with the activation of protein kinase A, resulting in airway muscle relaxation from the trachea to the end of bronchioles. LEV acts as an efficient agonist that relaxes the airway without a spasmogen, thereby protecting against all bronchoconstrictor disputes.

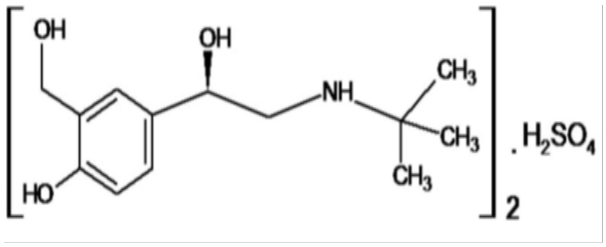

Figure 1: Levosalbutamol Sulphate

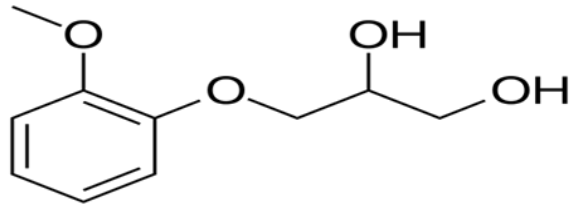

Figure 2: Guaiphenesin

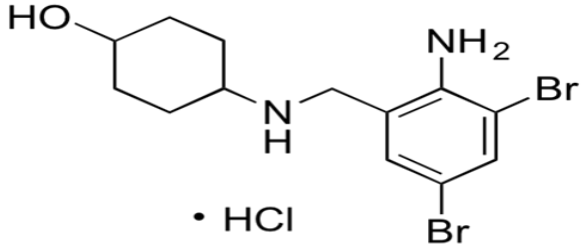

Figure 3: Ambroxol Hydrochloride

Guaiphenesin(GUA) / Guaifenesin/Glyceryl guaiacolate (Sethi PD, 1997; British Pharmacopoeia, 2008) (Fig.2) with Molecular formula $C_{10}H_{14}O_4$ and Molar mass: 198.216 g/mol is an expectorant medication generally taken by mouth to aid phlegm formation from the airways in acute respiratory tract infections(RTI). GUA is used as an expectorant by reducing the viscosity of secretions and increasing the volume in the tracheo-bronchi (Seagrave JC *et al.,* 2012; Prabhu Shankar S *etal.,* 2010). It also aids the flow of respiratory tract secretions, allowing ciliary progress to clutch the secretions upward towards the pharynx. As a result, it may enhance the effectiveness of the cough reflex and aid removal of the mucus secretions (Houtmeyers E *et al.,* 1999; Tripathi KD, 2008; Indian Pharmacopoeia, 2010).

Ambroxol Hydrochloride(AMB) (Fig.3) is an active mucolytic agent, Molecular formula $C_{13}H_{18}Br_2N_2O$ with Molecular mass of 378.1028 g/mol which is used in the treatment of respiratory diseases, pain relief in the acute sore throat (Sanderson RJ, 1976; Malerba and Ragnoli, 2008). AMB is a very potent neuronal Na+ channels inhibitor (WeiserT, 2006). LEV, GUA and AMB monographs are seen in Indian Pharmacopoeia (IP) (Indian Pharmacopoeia, 2006).

A literature survey reveals many techniques have been stated for the determination of LEV, GUA and AMB either alone or in combinations with other active ingredients in a multi-component tablet and liquid dosage formulation as predictable with the variation of column detector and mobile phase. Various methods like HPLC(Vasudevan M *et al.,* 2007; Joshi S *et al.,* 2011; Jain j *et al.,* 2008; Prathap S *et al.,* 2010; Mukesh M *et al.,* 2010; Krishnaveninagappan J *et al.,* 2008; Srividya P *et al.,* 2013; Laura C *et al.,* 1983; Vani R *et al.,* 2014) HPTLC (Prasant j, 2010; Sharma E, 2012; Krunal S, 2014; Bagada H, 2013), Spectrophotometric(Kim R *et al.,* 2011; Umadevi B *et al.,* 2009; Nehal S, 2012; Nirav C *et al.,* 2013; Amit P *et al.,* 2011; Gangwal S *et al.,* 1999) and few LC-MS(Harshal P *et al.,* 2014; Snehal G *et al.,* 2014; Dong X *et al.,* 2013) methods were used for determination of drugs even in Human plasma. Therefore, the present work is aimed to build up and validate RP UPLC method for simultaneous estimation of cough syrup containing LEV, GUA, and AMB in bulk and pharmaceutical dosage forms according to ICH guidelines (ICH Q2(R1) 1996). Hence, it has driven the authors to develop a method which is new, simple, fast, economical, precise, and accurate for the simultaneous estimation of all the three drugs in their pharmaceutical dosage forms which is better than the previously developed methods by HPLC.

Experimental

MATERIALS AND METHODS

Instrumentation: Chromatography has performed withUHPLC_Agilent_1220 Infinity LC with high-speed Auto Sampler with Open Lab_ Chemstation software using a UV detector at 276nm.

Reagents and Chemicals: Reference standards of Levosalbutamol, Guaiphenesin & Ambroxolwere provided as gift samples from Synthia Research Labs Private Limited, Pondicherry

Sample: Commercial syrup Manufactured by NTK Pharma, Chennai, India.

- PRODUCT NAME: EXIL LS
- BATCH No.: MNRB-02
- MFG DATE: FEB-2018
- EXP DATE: JAN-2020
- MANUFACTURED BY: NTK PHARMA

Chemicals: Acetonitrile, water, methanol, were procured from Merck, Mumbai, and potassium dihydrogen orthophosphate, procured from Rankem, Mumbai. All other solvents used in this research are of HPLC grade.

Chromatographic conditions

The mobile phase consisted of Buffer [2.7218 g of KH_2PO_4 in 1000 ml of water adjusted the pH to 3.0 with orthophosphoric acid], Methanol and Acetonitrile is taken in the ratio of 60:20:20 and with 0.8 ml/min flow rate. The solution was taken to vacuum filtration through a 0.45µm nylon membrane filter and pumped at ambient temperature. ODS (C18 4.6mm x 1000mm, 3.5µm) (Agilent - Zorbax Eclipse plus C18 – Rapid Resolution) was used as the stationary phase. LEV, GUA, and AMB have different λ_{max}, by considering the various chromatographic parameters for all the drugs. 2.0 µl was injected with 5.0 min run time at 276 nm using a UV detector. The retention time of LEV, GUA, and AMB was found to be 1.07min, 1.99min and 3.55 min respectively with a resolution of about 9.240. The resulting HPLC chromatogram was shown in (Fig.4).

Sample preparation

Preparation of Phosphate buffer: 2.722 gm of KH2PO4 was dissolved in one litre (1000ml) of HPLC grade water to get 0.02 M Phosphate buffer and the pH adjusted to 3.0 with Orthophosphoric acid. 0.45µm nylon membrane filter was used to filter the buffer to remove any gases or fine particles.

Preparation of the mobile phase

The Phosphate buffer 0.02M, Methanol and Acetonitrile HPLC grade were mixed in the ratios of 60:20:20 v/v, filtered through 0.45 µm membrane filter and degassed by sonicator.

Preparation of standard stock solution

Standard stock solutions of LEV, GUA, and AMB were prepared separately. An accurately weighed 12.5 mg of Levosalbutamol was transferred into a 100 ml volumetric flask and dissolved in methanol, the volume is made up to the volume with methanol. 250.0 mg GUA and 150.0 mg AMB was transferred to 100 ml volumetric flask separately and dissolved it with HPLC grade methanol and bath sonicated for 10 min to ensure complete solubilization. After 10 min, the volume was made to 100 ml with the same HPLC grade methanol. From this 100 ml solution 2 ml of LEV and 5 ml of GUA and AMB was taken out and the volume was made up to 25ml in 25ml volumetric flask with mobile phase which has a final concentration of 10 µg/mL of LEV, l, 500 µg/mL of GUA and 300 µg/ mL AMB of each reference standard.

Sample preparation

Take 5ml of suspension in a 100ml volumetric flask and 40ml of methanol was added and sonicated for 10 minutes, remaining volume made up to 100ml with mobile phase (55ml). All the three drugs are having a final concentration of 10µg/mL of Levosalbutamol, 500µg/mL of Guaiphenesin and 300µg/mL Ambroxol Hydrochloride.

RESULTS AND DISCUSSION

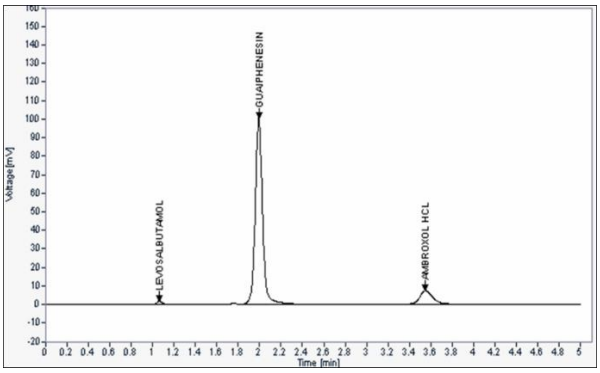

Figure 4: Developed chromatogram

Method development

Reverse phase HPLC separation was tried to build up new method with a range of mobile phases viz., Methanol and Water, Acetonitrile and Water, in which all the three drugs did not react properly, and the poor resolution was observed. The mobile phase was also examined for its organic content to optimise the separation of three drugs. To progress the tailing factor, the pH of the mobile phase becomes a key factor. At pH 3.0 all the three drugs eluted with better partition. Thereafter, Buffer: Methanol: ACN was considered in isocratic ratio: %buffer / %methanol / %ACN: 60/20/20, with a flow rate of 0.8mL/min was employed ODS (C18 4.6mm x 1000mm, 3.5µm) particle size was used as the stationary phase to develop resolution and the peaks tailing were condensed noticeably and brought close to 1. To investigate all the three drugs, detection was tried at different wavelengths from 205nm to 280nm. All the three drugs LEV, GUA, and AMB showed maximum absorption at 276nm with a UV detector. The attained chromatogram was shown in the (Fig.4).

Method Validation

The validation (ICH Q2(R1), 2005; Beckett and Stenlake, 1997) of the technique was carried out as per ICH guidelines and the factors evaluated were system suitability, accuracy & precision, Robustness, specificity, linearity, LOD and LOQ.

System suitability

As per the chromatographic conditions, the UHPLC method was optimised. To check the system suitability, one blank followed by 6 replicates of a single calibration standard solution of 10 µg/mL of LEV, l, 500 µg/mL of GUA and 300 µg/mL AMB was injected. The parameters viz., as retention time, theoretical plates, peak asymmetry and resolution were taken, and results were presented in (Table

1) to establish the system suitability for the planned method.

Specificity

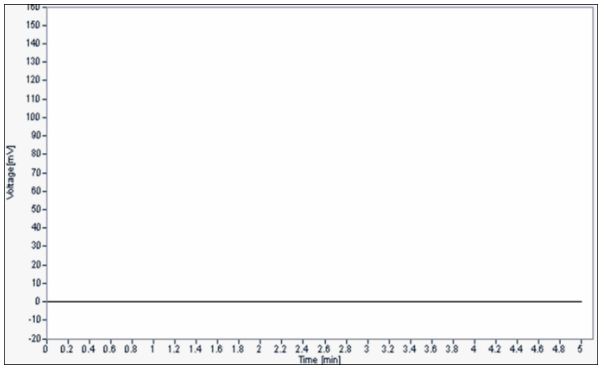

Figure 5: Blank

The excipients and other additives usually effect in the combined dosage form of LEV, GUA, and AMB for the determination under optimum conditions was examined. The specificity of the RP-UHPLC technique was recognized by injecting the blank and placebo solution into the UHPLC system. The chromatogram of blank was represented in (Fig .5), and the readings are shown in (Table 2).

Linearity

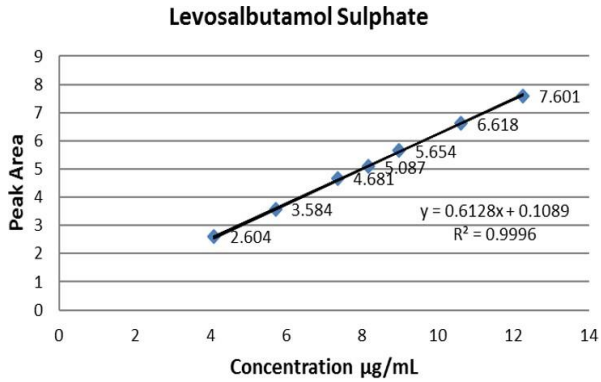

Figure 6: Linearity of Levosalbutamol Sulphate

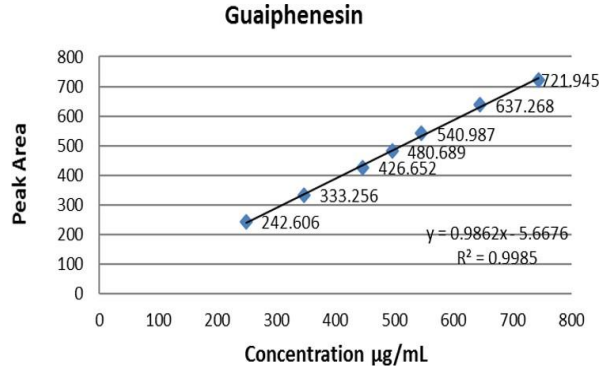

Figure 7: Linearity of Guaiphenesin

The linearity of response for LEV, GUA and AMB were between 2.604-7.601 µg/mL, 242.606-721.945 µg/mL and 33.073-96.107 µg/mL respectively. These responses were represented by a linear regression equation as follows: y (LEV) = 0.612x+0.108 (r^2=0.999), y (GUA) = 0.986x-5.667 (r^2=0.998) and y (AMB) = 0.215x+0.331 (r^2=0.998) and regression line was developed by least squares technique and correlation coefficient (r^2) for LEV, GUA and CAN is found to be greater than 0.98 and the curves established were linear, shown in Fig.6, 7 & 8) and in Table 3.

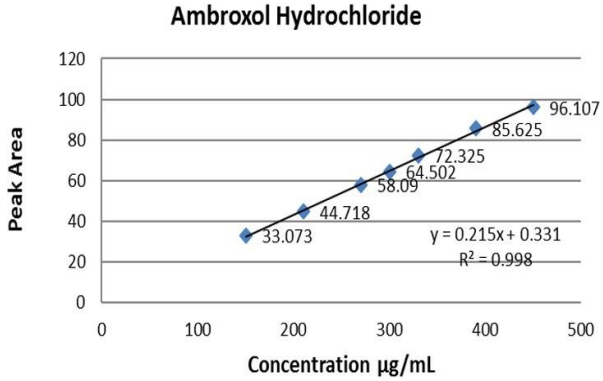

Figure 8: Linearity of Ambroxol Hydrochloride

Recovery

A definite concentration of standard drug (10%, 20% & 30 % level) was added, and recovery was studied to the pre-analyzed sample solution. The mean recovery percentage for LEV, GUA, and AMB are 100.39, 99.99 and 100.3 respectively and these results are within the tolerable limit of 98-102. The % RSD for LEV, GUA, and AMB are 1.41, 0.43 and 0.74

Respectively and %RSD is within the limit of ≤ 2. Therefore, the projected method is accurate, and the results were summarized in Table 4, 5& 6.

Accuracy and Precision: Validation method concerning accuracy by replicates injection of standard solution at low, medium, and high concentration levels. Precision states the closeness of conformity between the series of measurements obtained from multiple sampling of same homogenous samples under approved conditions. Six replicates injections in the same concentration were analyzed in the same day for repeatability and the % RSD for LEV, GUA, and AMB found to be 1.08, 0.49 and 0.69 respectively as all the statistical results were within the range of acceptance, i.e. % RSD less than 2.0 and S.D less than 1.0. % and hence the method is reproducible, and the results are shown in Table 7& 8.

Robustness

The robustness was recognized by the composition of the mobile phase, changing the flow rate and change in wavelength within allowable limits from actual chromatographic conditions. It was experiential that there was no noticeable change in mean Rt and RSD within a limit of ≤ 2. The tailing and resolution factor and theoretical plate numbers are found to be satisfactory limits for LEV, GUA, and AMB. Thus, the method is reliable with variations

Table 1: System suitability

S.No	Parameter	LEV*	GUA*	AMB*
1.	Rt	1.07	1.99	3.55
2.	Theoretical plates	2446.502	4622.468	4180.347
3.	Tailing factor	1.08	1.078333	1.22
4.	Area%	1.035	87.20333	11.76833
5.	SD	0.10	0.58	0.29
6.	%RSD	1.78	0.54	0.45

*mean average of Six determinations

Table 2: Specificity

SNo.	Injection	LEV Rt	Area	GUA Rt	Area	AMB Rt	Area	SD	%RSD
1.	LEV(6)	1.07	6.020	0.00	0.00	0.00	0.00	0.060	1.000
2.	GUA(6)	0.00	0.00	2.00	549.00	0.00	0.00	2.62	0.48
3.	AMB(6)	0.00	0.00	0.00	0.00	3.66	73.750	0.7	0.95
4.	Blank	About1.07	NIL	About2.0	NIL	About3.6	NIL	NIL	NIL
5.	Placebo	About1.07	NIL	About2.0	NIL	About3.6	NIL	NIL	NIL

Table 3: Linearity

S. No	Linearity of LEV		Linearity of GUA		Linearity of AMB	
	Conc (µg/ml)	Peak area	Conc (µg/ml)	Peak area	Conc (µg/ml)	Peak area
1	4.09	2.604	247.94	242.606	150.19	33.073
2	5.72	3.584	347.11	333.256	210.27	44.718
3	7.36	4.681	446.28	426.652	270.35	58.09
4	8.17	5.087	495.87	480.689	300.39	64.502
5	8.99	5.654	545.46	540.987	330.43	72.325
6	10.63	6.618	644.63	637.268	390.51	85.625
7	12.26	7.601	743.81	721.945	450.58	96.107
Slope	0.612		0.986		0.215	
Y-Intercept	0.108		5.667		0.331	
Co-Relation Co-Efficient	0.999		0.998		0.998	

Table 4: Recovery of Levosalbutamol Sulphate

S.No	Sample ID	Standard Area	Sample Area	Assay obtained in Accuracy Test, Avg of 9 Determinations	Std Spiked (mg)
1	Spiked with 10%	5.634	6.028	0.997	0.100
2	Spiked with 20%	5.634	6.579	0.997	0.200
3	Spiked with 30%	5.634	7.183	0.997	0.300

Table 4: Recovery of Levosalbutamol Sulphate (Contd….)

S.No	Assay obtained mg	Amount Recovered mg	Recovery %= (Amount recovered)/(Std amount spiked) x 100
1	1.098	0.101	101.00
2	1.196	0.199	99.50
3	1.299	0.302	100.67

Table 5: Recovery of Guaiphenesin

S.No	Sample ID	Standard Area	Sample Area	Assay obtained in Accuracy Test, Avg of 9 Determinations	Std Spiked (mg)
1	Spiked with 10%	510.388	548.190	49.835	5.002
2	Spiked with 20%	510.388	599.738	49.835	10.005
3	Spiked with 30%	510.388	651.688	49.835	15.007

in the analytical situations and the results of LEV, GUA, and AMB were shown in Table 9, 10 & 11.

Limit of Detection and Limit of Quantification

The LOD can be described as the negligible level of analytes that gives a considerable reaction, and LOQ was analyzed as the lowest amount of analytes that was quantified reproducibly. Based on the standard deviation of the response and the

Table 5: Recovery of Guaiphenesin (Contd …)

S.No	Assay obtained mg	Amount Recovered mg	Recovery %= (Amount recovered)/(Std amount spiked) x 100
1	54.854	5.019	100.34
2	59.869	10.034	100.29
3	64.743	14.908	99.34

Table 6: Recovery of Ambroxol Hydrochloride

S.No	Sample ID	Standard Area	Sample Area	Assay obtained in Accuracy Test, Avg of 9 Determinations	Std Spiked (mg)
1	Spiked with 10%	68.131	71.970	29.986	3.002
2	Spiked with 20%	68.131	78.644	29.986	6.003
3	Spiked with 30%	68.131	85.641	29.986	9.005

Table 6: Recovery of Ambroxol Hydrochloride (Contd…)

S.No	Assay obtained mg	Amount Recovered mg	Recovery %= (Amount recovered)/(Std amount spiked) x 100
1	33.012	3.026	100.80
2	35.988	6.002	99.98
3	39.002	9.016	100.12

Table 7: Accuracy

S. No	Sample ID	Levo Salbutamol Sulphate		Guaiphenesin		Ambroxol Hydrochloride	
		in mg	in %	in mg	in %	in mg	in %
	LOW -SPL.-01	1.001	100.10	49.454	98.91	29.714	99.05
1.	LOW -SPL.-02	0.998	99.80	49.740	99.48	29.584	98.61
	LOW -SPL.-03	1.010	101.00	50.075	100.15	30.040	100.13
	MID. -SPL.-01	0.991	9.10	49.617	99.23	29.942	99.81
2.	MID. -SPL.-02	0.994	99.40	49.596	99.19	29.849	99.50
	MID. -SPL.-03	1.001	100.10	49.542	99.08	30.414	101.38
	HIGH -SPL.-01	1.003	100.30	50.066	100.13	30.179	100.60
3.	HIGH -SPL.-02	0.985	98.50	50.506	101.01	30.257	100.86
	HIGH -SPL.-03	0.990	99.00	49.916	99.83	29.893	99.64
	Average :	1.00	99.70	49.835	99.67	29.986	99.95
4.	SD :	0.01	0.77	0.55	0.68	0.26	0.88
	% RSD :	1.00	0.77	1.10	0.68	0.87	0.88

Table 8: Precession

S.No.	Sample ID	Levo Salbutamol Sulphate		Guaiphenesin		Ambroxol Hydrochloride	
		in mg	in %	in mg	in %	in mg	in %
1.	SPL. -01	1.000	100.00	50.393	100.79	30.305	101.02
	SPL. -02	0.984	98.40	50.348	100.70	30.316	101.05
	SPL. -03	0.994	99.40	50.151	100.30	30.320	101.07
2.	SPL. -04	1.001	100.10	50.534	101.07	30.311	101.04
	SPL. -05	0.997	99.70	50.499	101.00	29.968	99.89
	SPL. -06	1.017	101.70	50.891	101.78	29.864	99.55
3.	Average :	0.999	99.88	50.469	100.94	30.181	100.60
	SD :	0.01	1.08	0.25	0.49	0.21	0.69
	% RSD :	1.00	1.08	0.50	0.49	0.70	0.69

Table 9: Robustness of Levosalbutamol Sulphate

S.No.	Parameter	Drug (LEV)	Avg Peak Area	SD	% RSD
1.	Flow rate	5% decrease	6.290	0.12	1.91
		5% Increase	4.940	0.08	1.62
2.	Mobile phase Change	More organic	6.910	0.13	1.88
		More Aqueous	5.350	0.03	0.56
3.	Change in Wavelength	+2 nm	5.830	0.08	1.37
		-2nm	5.340	0.10	1.87

slope, these two parameters were considered using the formula. LOD and LOQ were calculated using equation LOD=3.3×s/S and OQ=10×s/S, where s= standard deviation of Y-intercept,
S = average slope of calibration curve. LOD and LOQ for LEV, GUA, and AMB were 0.166µg/mL, 0.5809 µg/mL, 0.2479 µg/mL, 0.9917 µg/mL and 1.5062, 6.0250 µg/mL respectively.

CONCLUSION

A novel trouble-free precise, accurate and validated RP-UPLC method has been built for simultaneous estimation of Levosalbutamol Sulphate(LEV), Guaiphenesin(GUA) and Ambroxol Hydrochloride(AMB) with good retention time, economical mobile phase, and quick run time. Therefore, it can be applied for regular quality control of syrups, multicomponent tablets in QC laboratories and industries and is used for the usual analysis in both bulk and pharmaceutical dosage forms.

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