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DEVELOPMENT AND VALIDATION OF RP-HPLC METHOD FOR THE ESTIMATION OF DOXOFYLLINE AND TERBUTALINE IN PURE AND IN ITS FORMULATION

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ABSTRACT: A new simple, rapid, and sensitive reversed-phase liquid chromatographic method was developed for the estimation of doxofylline and terbutaline in the tablet dosage form. The chromatographic separation was achieved on ODS C_{18}column (150 × 4.6 mm, 5 µm) at ambient temperature and effluent monitored at 257 nm. The mobile phase consists of ammonium acetate buffer (pH adjusted to 3 with o-phosphoric acid) and acetonitrile in the ratio of 50:50 v/v. The flow rate was maintained at 1 ml/min. The method was validated concerning linearity, precision, accuracy, ruggedness, limit of detection, limit of quantification and robustness. The assay methods were found to be linear from 16-96 µg/ml for doxofylline and 0.2-1.2 µg/ml for terbutaline. All validation parameters were within the acceptable range. The mean recovery was 99.35 and 99.25 for doxofylline and terbutaline respectively. The % RSD value was found to be less than 2. The limit of detection and limit of quantification for doxofylline and terbutaline were found to be 0.06 µg/ml and 0.024 µg/ml and 0.21µg/ml and 0.079 µg/ml respectively. The result of the study showed that the proposed RP-HPLC method for the simultaneous estimation of doxofylline and terbutaline in the tablet dosage form is simple, accurate, sensitive, precise, specific and rapid which is useful for routine analysis of doxofylline and terbutaline in its formulations.

INTRODUCTION: Terbutaline (TBT) is chemically recognized as (±)-α-[(tert-butylamino) methyl]-3,5-dihydroxybenzyl alcohol, and its molecular weight is 548.65 g/mol. It is a white to gray-white crystalline powder and soluble in water, 0.1N HCl, slightly soluble in methanol, insoluble in chloroform. It's melting point is 119-122 ºC. TBT **Fig. 1** is used to relieve and prevent bronchospasms caused by asthma, emphysema or bronchitis. It produces bronchodilation by relaxing bronchial smooth muscle through β2 receptor stimulation. It also decreases uterine contractility and may be used to arrest premature labor. Current asthma guidelines recommend that inhaled short-acting beta 2 agonists such as TBT be used on an 'as-required,' not regular, basis. In those patients requiring more than occasional use of TBT, anti-inflammatory therapy is also needed. An increased requirement for, or decreased duration of effect of, Terbutaline indicates deterioration of asthma control and the need for increased anti-inflammatory therapy [1, 5].

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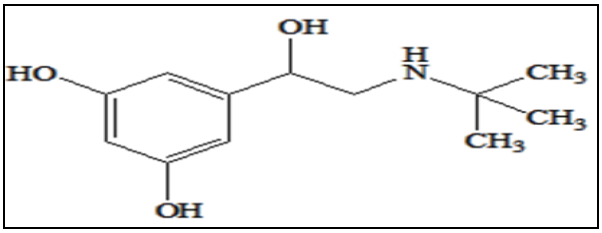

FIG. 1: STRUCTURE OF TERBUTALINE

Doxofylline (DOX) is chemically recognized as 7-(1,3-Dioxolan-2-yl methyl) theophylline), is a new generation of long-acting oral methylxanthine derivative and its molecular weight is 266g/mol. It is a white crystalline powder and soluble in water, chloroform, acetone and benzene, and it's melting point is 144-145 ºC. DOX **Fig. 2** is used to treat Asthma and Chronic Obstructive Pulmonary Disease (COPD) [2, 5]. Doxofylline mechanism of action is related to the inhibition of phosphodiesterase activities within the smooth muscle cells and thereby causes smooth muscle relaxation and thus suppressing asthma [3].

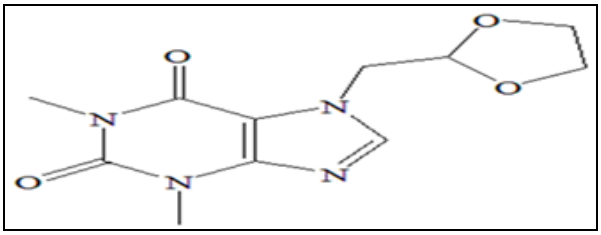

FIG. 2: STRUCTURE OF DOXOFYLLINE

In February 2009, a fixed-dose combination of Doxofylline and Terbutaline sulfate was approved by DCG (I) in India. Co-administration of Doxofylline with Terbutaline sulfate gives better bronchodilation with a lower degree of skeletal muscle tremor than a higher dose of terbutaline sulfate by mouth alone. Therefore, a fixed-dose combination of Doxofylline and Terbutaline sulfate is a better alternative for the treatment of acute and chronic asthma [4].

Based on a thorough review of literature only one single HPLC [5] and two UV Spectroscopic [4, 6] analytical methods have been reported for the quantification of DOX and TBT in pure and combined pharmaceutical dosage. Apart from this, there are many literature reviews were found in various analytical techniques for the estimation of DOX and TBT alone. This includes, analysis of Doxofylline and Terbutaline sulphate, exerts a prophylactic effect against bronco constriction and pleurisy induced by PAF [7], spectrophotometric determination of doxofylline in tablet formulation [8-11], development and validation of a sensitive LC-MS/MS method with Electrospray Ionization for quantitation of Doxofylline in human serum [12], method development and degradation studies of Doxofylline by RP-HPLC and LC-MS/MS [13-14], development and validation of a stability-indicating RP-HPLC method for analysis of Doxofylline in Human Serum [15], non-extraction HPLC method for simultaneous measurement of Dyphylline and Doxofylline in serum [16], simultaneous estimation of Doxofylline and its combinations by RP-HPLC method from solid dosage forms [17-22], HPTLC methods for determination of Doxofylline in bulk and formulations [23-24].

There are some other literature review showed the estimation of these drug in combination with another drug formulation by different analytical methods such as, spectrophotometric simultaneous analysis of Ambroxol Hydrochloride, Guaifenesin and Terbutaline Sulphate in liquid dosage form such as syrup [25], RP-HPLC and stability indicating HPLC methods for simultaneous determination of Terbutaline alone and with combinations [26-29], simultaneous determination of Terbutaline Sulphate with other drugs in tablet formulation by UV Spectrophotometry [30], stability indicating HPTLC method for determination of Terbutaline Sulphate in bulk and from submicronised dry powder inhalers [31]. Thus, this present study report deals with the simultaneous estimation of Doxofylline and Terbutaline Sulphate by HPLC in bulk drug and tablet dosage form. The projected technique was validated as per the International Conference on Harmonisation guidelines [32, 33].

MATERIALS AND METHODS:

Equipment: The chromatographic technique performed on an LC -Sol, VP Shimadzu with a UV detector, reversed phase ODS C18 column (150 × 4.6 mm, 5 µ) as a stationary phase, AUY 220 (Shimadzu) analytical balance, ultrasonic bath sonicator was used in this study.

Materials: MucomaT (Doxofylline 400 mg and terbutaline 5 mg) was obtained from U Win Life Sciences, Malappuram, Kerala. Doxofylline and Terbutaline (API) were obtained as a gift sample. HPLC grade Ammonium acetate, o-phosphoric acid, acetonitrile were obtained from Merk, Mumbai. All the chemicals used were of A.R. Grade.

Chromatographic Conditions: The sample separation was achieved on an ODS C18 (150 × 4.6 mm, 5 µ) column, aided by mobile phase mixture of Ammonium acetate buffer (pH adjusted to 3

with o-phosphoric acid: acetonitrile (50:50 v/v)). The flow rate was 1 ml/min with the injection volume as 20 µl at ambient temperature and was detected on a UV detector at a wavelength of 257 nm.

Buffer Preparation: Weighed about 2.83 g of ammonium acetate dissolved in 1000 ml of HPLC grade Water. This solution is mixed well and adjusted the pH to 3 ± 0.2 with dilute orthophosphoric acid (OPA) solution. After that, filter the solution through a 0.45 µ membrane filter.

Mobile Phase Preparation: Prepared a mixture of ammonium acetate buffer solution (pH-3.0) and acetonitrile in the ratio of 50:50 v/v respectively, mixed well and sonicated for 10 min.

Standard Solution Preparation: The stock solutions of DOX and TBT were prepared by accurately weighing 40 mg DOX and 0.5 mg TBT in a 50 ml volumetric flask and dissolve it in 30 ml mobile phase, and it was sonicated for 5 min and diluted to 50 ml with mobile phase.

Sample Solution Preparation: The working standard solutions of DOX and TBT were prepared by accurately transferring 0.2, 0.4, 0.6, 0.8, 1 and 1.2 ml aliquots to 10 ml volumetric flask and were made up to mark with mobile phase to obtain concentration of is 16-96 µg/ml for DOX and 0.2-1.2 µg/ml for TBT respectively.

Determination of Working Wavelength (λ_{max}): Weighed and transferred both the drugs terbutaline and doxofylline working standard into a 50 ml volumetric flask, added about 10 ml of methanol to dissolve and volume is made up to 50 ml with methanol. Above solution is scanned in the range of 200 nm to 400 nm. The λmax was found to be 257 nm.

RESULTS:

Method Development and Validation: After the several initial trials with the mixtures of water, methanol, acetonitrile, potassium dihydrogen, o-phosphoric acid and buffers in various combinations and proportions, a trial with mobile phase mixture of ammonium acetate buffer and acetonitrile (50:50), the flow rate of 1 ml/min provide a sharp peak. The chromatogram was shown in **Fig. 3**.

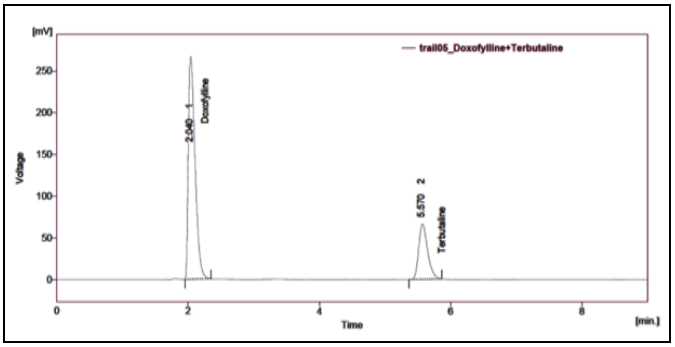

FIG. 3: A TYPICAL CHROMATOGRAM FOR DOX AND TBT

Linearity: A series of solutions were prepared using DOX and TBT working standard at concentration levels from 20% to 120% of assay test concentration and each solution was injected into HPLC as per methodology (20%, 40%, 60%, 80%, 100%, and 120%). Calibration curves were constructed by plotting peak area *vs*. concentration of DOX and TBT, and the regression equation was calculated. The calibration curves were plotted over the concentration range of 16-96 µg/ml for DOX and 0.4-1.2 µg/ml for TBT. From the standard stock solution of a mixture of DOX and TBT, (0.2, 0.4, 0.6, 0.8, 1 and 1.2 ml) aliquots were taken into 10 ml volumetric flask and diluted up to mark with mobile phase. Aliquots (20 µL) of each solution were injected under the operating chromatographic condition described above.

TABLE 1: LINEARITY DATA FOR DOX AND TBT

S. no.	DOX		TBT	
	Conc. (µg/ml)	Area (mV. sec) (n=6)	Conc. (µg/ml)	Area (mV. sec) (n=6)
1	16	391.222	0.4	134.936
2	32	794.883	0.8	281.806
3	48	1278.733	1.2	430.687
4	64	1690.916	1.6	563.173
5	80	2111.891	2.0	682.895
6	96	2510.841	2.4	811.054

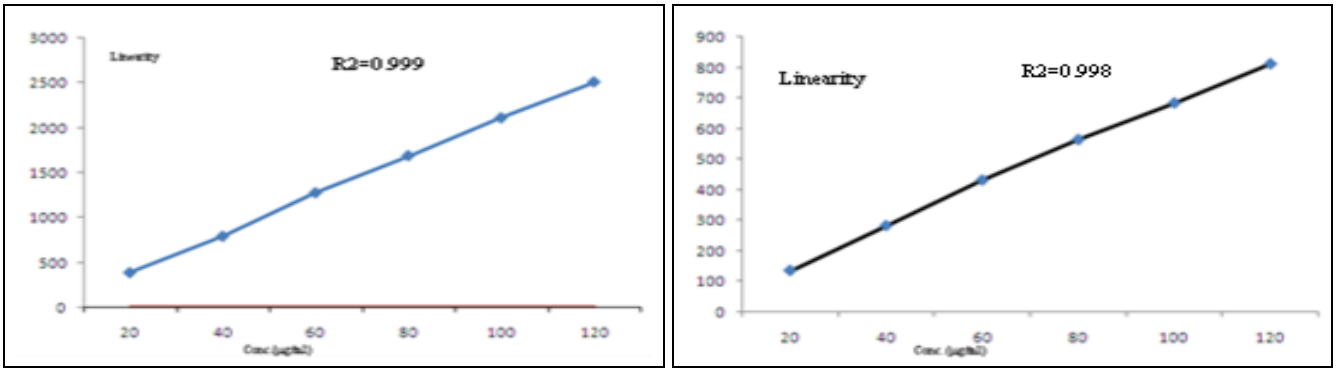

FIG. 4: LINEARITY PLOT FOR DOX AND TBT

Precision:

System Precision: The standard solution was prepared as per the test method and injected five times into the chromatographic system.

Method Precision: Prepared five sample solution as per the test method and injected each the solution into the chromatographic system **Table 2** and **Fig. 5**.

TABLE 2: SYSTEM PRECISION FOR DOX AND TBT

S. no.	System Precision				Method Precision			
	DOX (80 µg/ml)		TBT (1.0 µg/ml)		DOX (80 µg/ml)		TBT (1.0 µg/ml)	
	Rt (min)	Peak Area (Mv. Sec)	Rt (min)	Peak Area (Mv. Sec)	Rt (min)	Peak Area (Mv. Sec)	Rt (min)	Peak Area (Mv. Sec)
1	2.06	2136.082	5.65	370.127	2.1	2110.921	5.763	356.246
2	2.06	2136.903	5.66	368.213	2.13	2123.007	5.723	387.274
3	2.08	2115.853	5.67	351.190	2.103	2122.828	5.777	403.323
4	2.09	2125.393	5.66	340.214	2.107	2133.756	5.763	408.614
5	2.08	2102.381	5.67	339.010	2.117	2120.005	5.723	414.818
Mean	2.07	2123.811	5.66	370.196	2.1114	2122.103	5.7498	403.855
SD	0.0122	14.6347	0.0487	2.6783	0.01222	8.16355	0.02512	4.52357
% RSD	0.578	0.689	0.854	0.399	0.5787	0.3847	0.4369	0.6445

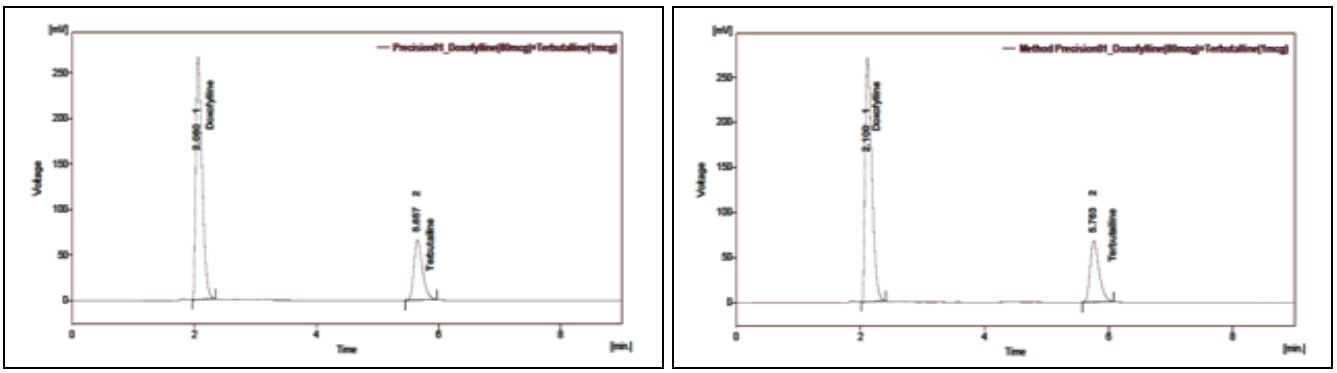

FIG. 5: CHROMATOGRAM FOR SYSTEM AND METHOD PRECISION OF DOX (80 µg/ml) AND TBT (1.0 µg/ml)

Ruggedness: The ruggedness of the test method was studied by analyzing the sample by two analysts. The % RSD of the assay values between the two analysts was calculated in **Table 4**.

TABLE 3: INTERMEDIATE PRECISION FOR DOX AND TBT

Drug	Analyst-1		Analyst-2	
	Rt. (min)	Area (Mv. sec)	Rt. (min)	Area (Mv. sec)
DOX	2.05	75.238	2.05	75.262
TBT	5.62	24.762	5.60	24.738

Accuracy (Recovery): The accuracy of the method was determined by calculating the recovery of DOX and TBT by the standard addition method. The known amounts of standard solutions of DOX and TBT were added to pre quantified sample solutions of DOX and TBT equivalent to 80%, 100%, and 120% of the labeled amount as per the test method. The closeness of the obtained value to the true value indicates that the proposed method is accurate **Table 4**.

$$\% \text{ Recovery} = [(ct - cu)/ ca] \times 100$$

TABLE 4: ACCURACY DATA FOR DOX AND TBT

Drug name	Pre analysed Sample Concentration (µg/ml)	Spiked amount (µg/ml)	Amount Recovered (µg/ml)	% Recovery
DOX	64	8	71.651	99.51
	80	8	87.140	99.02
	96	8	103.501	99.52
TBT	0.8	0.1	0.892	99.14
	1.0	0.1	1.092	99.33
	1.2	0.1	1.290	99.25

Robustness: To demonstrate the robustness of the method, prepared solution as per the test method injected at different variable conditions like using different conditions like flow rate and wavelength **Table 5** and **6**.

TABLE 5: RESULTS OF ROBUSTNESS STUDY FOR DOX AND TBT

Parameter	Modification	% RSD (n = 3)		Tailing factor	
		DOX	TBT	DOX	TBT
Flow rate (ml/min)	0.9	1.40	1.58	1.409	1.889
	1.1	1.81	0.83	1.263	1.781
Wavelength (nm)	255	1.03	1.67	1.350	1.789
	259	1.03	1.62	1.350	1.794

Limit of Detection and Limit of Quantification: The limit of detection (LOD) and limit of quantification (LOQ) were separately determined based on the standard deviation of the y-intercept and the slope of the calibration curve. The LOD and LOQ for DOX and TBT were found to be 0.06 µg/ml and 0.024 µg/ml and 0.21 µg/ml and 0.079 µg/ml respectively.

Analysis of Commercial Pharmaceutical: The proposed validated method was successfully applied to determine DOX and TBT in their tablet dosage form. The result obtained for DOX and TBT was comparable with the corresponding labeled amounts. 20 tablets of DOX (400 mg) and TBT (5 mg) were weighed and powdered and transfer it into 100 ml volumetric flask which contains 60 ml of methanol. The resulting solution was centrifuged at 3000 rpm for 5 min. The supernatant liquid contains 4000 µg/ml of DOX, and 50 µg/ml of TBT is filtered through a 0.45µ membrane filter. Then, the sample solutions are diluted to 4 µg/ml and 5 µg/ml by using a methanol solvent. A 20 volume of sample solution is injected into the HPLC system for six times **Table 6**.

TABLE 6: PERCENTAGE ASSAY RESULTS

Brand name	Content	Amount found (mg/tablet)	% Assay (n = 6)	% RSD (n = 6)
Mucosma T	Doxofyline (400 mg)	396.8	99.19	0.97
	Terbutaline (5 mg)	5.05	101	0.46

DISCUSSION: A RP-HPLC method was developed and validated for the determination of DOX and TBT in tablet dosage forms on ODS C18 (150 × 4.6 mm, 5 µm) with wavelength detection at 257 nm. The retention time of DOX and TBT was 2.68 min and 5.57 min respectively. A linear correlation was obtained between area and concentration of DOX and TBT in the concentration range of 16-96 µg/ml and 0.4-2.4 µg/ml respectively. The low RSD value of inter-day and intra-day at 257 nm, reveal that the proposed method is precise. The limit of detection (LOD) and limit of quantification (LOQ) for DOX and TBT were found to be 0.06 µg/ml and 0.024 µg/ml and 0.21 µg/ml and 0.079 µg/ml respectively. These data show that the method is sensitive for the determination of DOX and TBT.

The recovery experiment was performed by the standard addition method. The mean recoveries were found to be 99.35 and 99.25 for DOX and TBT respectively. The results of recovery studies indicate that the proposed method is highly accurate. The proposed method was found to be robust enough (% RSD < 2) to withstand such slight changes and allow routine analysis of the sample. The proposed validated method was successfully applied to determine DOX and TBT in

the tablet dosage form. The results obtained for DOX and TBT were comparable with the corresponding labeled amounts. There is no interference of the excipients with the absorbance of interest appeared. The mean % Assay was 99.19 and 101 for DOX and TBT respectively. Hence the proposed method is applicable for the routine simultaneous estimation of DOX and TBT in the pharmaceutical dosage form.

CONCLUSION: The results of the analysis of pharmaceutical formulation by the proposed method are highly reproducible and reliable, and it is in good agreement with the label claim of the drug. The method can be used for the routine analysis of the DOX and TBT in combined dosage form without any interference of excipients. From the results, it was found that the developed RP-HPLC method was found to be simple, accurate, sensitive, precise, specific and rapid. The method can be applied for routine analysis of DOX and TBT in pure and its formulations.

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CONFLICT OF INTEREST: The authors declare no conflicts of interest.

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