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Method development, validation and stability study for simultaneous estimation of Etofylline and Theophylline by RP-HPLC chromatography in marketed formulation

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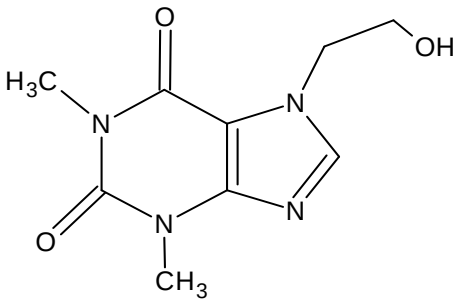
ABSTRACT

An approach of forced degradation study was successfully applied for the development of a stability-indicating assay method for simultaneous estimation of Etofylline and Theophylline in a formulation in the presence of its degradation products. The method showed adequate separation of Etofylline and Theophylline from their associated main impurities and degradation products. Separation was achieved on an YMC Pack-ODS-AQ, 150 x 4.6 mm the mobile phase 10mM Potassium Di-Hydrogen Phosphate : Acetonitrile (90:10) pH-4.5 with ortho phosphoric acid buffer flow rate of 1 mL/min and UV detection at 272 nm. Comprehensive stress testing of Etofylline and Theophylline Rt= 6.4 & 5.2 min was according to the International Conference on Harmonization (ICH) guideline Q1A (R2). The method was validated in terms of system suitability, precision, linearity, accuracy, robustness, ruggedness, LOD, LOQ and solution stability.

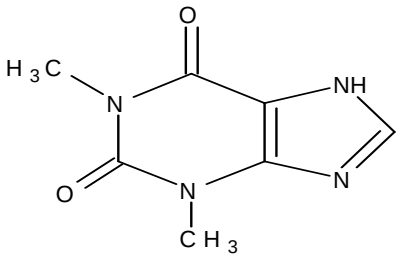
Keywords: Etofylline, Theophylline, RP-HPLC, Validation.

INTRODUCTION

Theophylline has maintained an important role as a potent and useful bronchodilator. However the use of theophylline is often restricted by its narrow therapeutic range. Etofylline is a bronchodilator and is normally applied in combination with theophylline. The pharmacological actions of etofylline are generally considered like those of theophylline. Unlike other xanthine derivatives, etofylline does not convert into theophylline in the body. This offers a wide therapeutic window and combination of etofylline and theophylline exhibits less frequent adverse side effects than an equivalent dose of theophylline alone. Etofylline is a Xanthine bronchodilator. Chemically known as 3,7-Dihydro-7-(2-hydroxyethyl)-1,3-dimethyl-1*H*purine-2,6-dione, the molecular structure of Etofylline is shown in Figure 1



Theophylline is a Xanthine bronchodilator. Chemically known as 1,3-dimethyl-7H-purine-2,6-dione, the molecular structure of Etofylline is shown in Figure 2



Simultaneous Etofylline and Theophylline is not official in any Pharmacopoeia. Literature study reveals that a UV and HPLC method and individual are available for estimation of Etofylline and Theophylline. Moreover there is no Simultaneous estimation of estimation of Etofylline and Theophylline and its formulations.

The objective of this work was to develop inexpensive, simple and rapid stability indicating RP-HPLC methods which would be accurate and precise.

The methods were validated according to ICH guidelines. The linearity of response, accuracy, and intermediate precision of the described methods has been validated.

EXPERIMENT AL SECTION

Etofylline and Theophylline were provided as a gift sample by Suven Pharmaceutical Pvt Ltd. Hyderabad, India and its claimed purity was 99.0%. and Marketed formulation sample Deriphylline Tablets (Etofylline 77mg and Theophylline 23mg) claim Cadila Healthcare Limited (Sikkim, India).

All other reagent required for experimentation was of analytical reagent (AR) grade. Chemicals used for this experiment were, Methanol (HPLC grade) were purchased from fisher scientific pvt. Ltd, Acetonitrile (HPLC grade) was purchased from Spectrochem pvt. Ltd, Mumbai, ortho phosphoric acid (AR grade) was purchased from fisher scientific pvt. Ltd, Potassium Dihydrogen Phosphate (AR grade) is Merck, pvt. Ltd.

Equipments

Analysis was performed on a chromatographic system Agilent 1200 series equipped with an auto injector, Diode array detector and a single-beam Agilent UV-Visible spectrophotometer, Model 8453.

Liquid chromatographic conditions

Chromatographic conditions were obtained using a stainless steel column YMC Pack-ODS-AQ, 150 mm × 4.6 mm 5µm), which was maintained at 30° C. The analytical wavelength was set at 272 nm and samples of 5µl were injected to HPLC system. The mobile phase was 10mM Potassium Di-Hydrogen Phosphate : Acetonitrile (90:10) pH-4.5 with ortho phosphoric acid at a flow rate of 1.0ml/min. Diluent as a water. The mobile phase was filtered through 0.45µm filter (Sartorius, Germany) and degassed for 10 minutes by sonication.

Preparation of Standard Solution

The standard stock of Etofylline and Theophylline was prepared by dissolving 100mg and 30 mg of working standard in water in 100 mL volumetric flask. After sonicate for 5 min and volume was made up to the mark. 5 mL aliquot from the standard stock solution of Etofylline and Theophylline was transferred in 50 mL volumetric flask, and the volume was made up to the mark with diluent.

Assay Sample preparation:

Twenty tablets were weighed, their mean weight was determined, and they were crushed in a mortar. An amount of powdered mass equivalent to Etofylline 100 mg and Theophylline equivalent to 30 mg weighed, add water, sonicate for 15 min and make upto mark with diluent in 100ml volumetric flask. 5 mL aliquot from the stock solution of sample was transferred in 50 mL volumetric flask, and the volume was made up to the mark with diluent. Filtered it through 0.45µ (PVDF Millipore Filter).

Stress Degradation studies:

Acid Degradation: Treated with 20ml 1 N HCl and heated on boiling water bath for 3 hours then cool at room temperature afte that add 20ml 1 N NaOH for neutralize the solution.

Alkali degradation: Treated with 20ml 1 N NaOH and heated on boiling water bath for 3 hours then cool at room temperature afte that add 20ml 1 N HCl for neutralize the solution.

RESULTS AND DISCUSSION

The solution 10 µg/ml of Etofylline and Theophylline were scanned in the UV range of 200- 400 nm and their Wavelength was found to be 272 respectively and Etofylline and Theophylline showed very good absorbance at this wavelength. Literature review reveals only individual methods for estimation of Etofylline and Theophylline but no methods were reported for simultaneous estimation of Etofylline and Theophylline. A simple, precise, accurate, RP-HPLC method has been developed for the estimation of Etofylline and Theophylline in bulk and in Tablet formulation. A Chromatogram of Etofylline and Theophylline shown in fig-1, Etofylline and Theophylline with retention time of 6.4 min and 5.2 min respectively.

Parameter	Etofylline	Theophylline
Retention Time	6.4	5.2
Symmetry	0.83	0.81
Plates	12409	12185
Resolution	5.42	
Selectivity	1.22	

Table 1 Performance Parameters of chromatogram

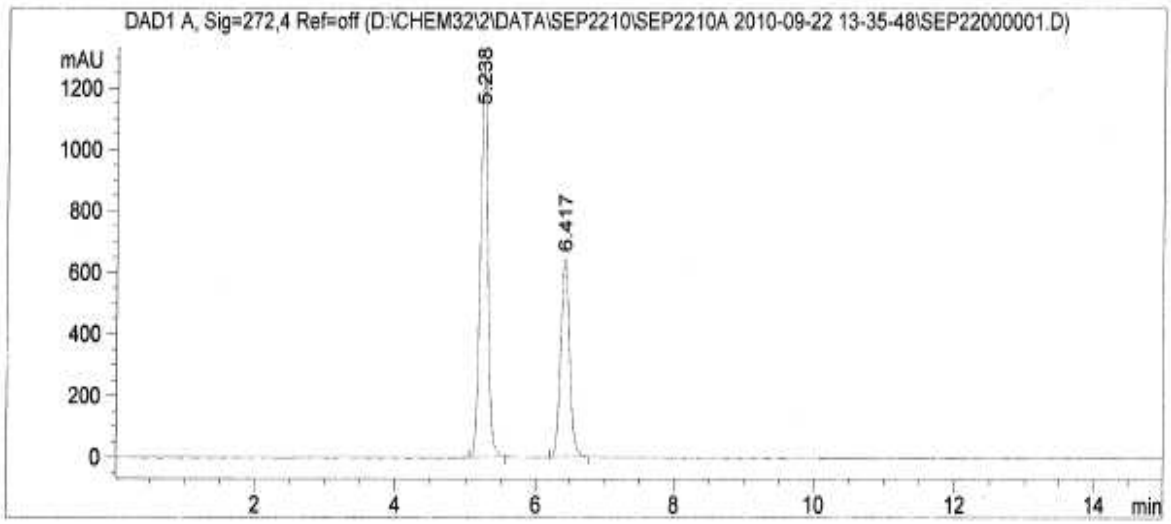


Figure 1 Chromatogram Of Etofylline and Theophylline

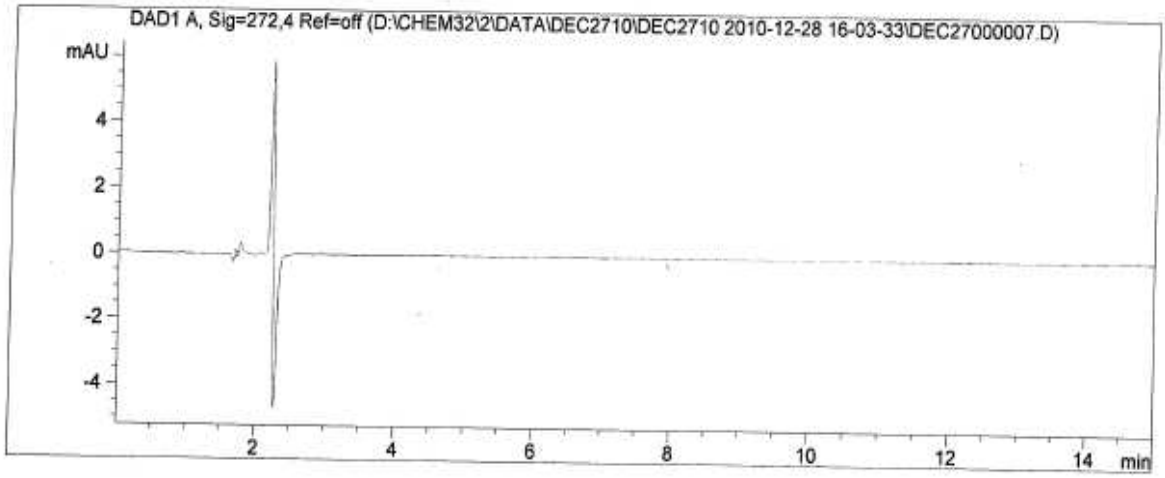


Figure 2 Chromatogram Of Acid Degradation (blank)

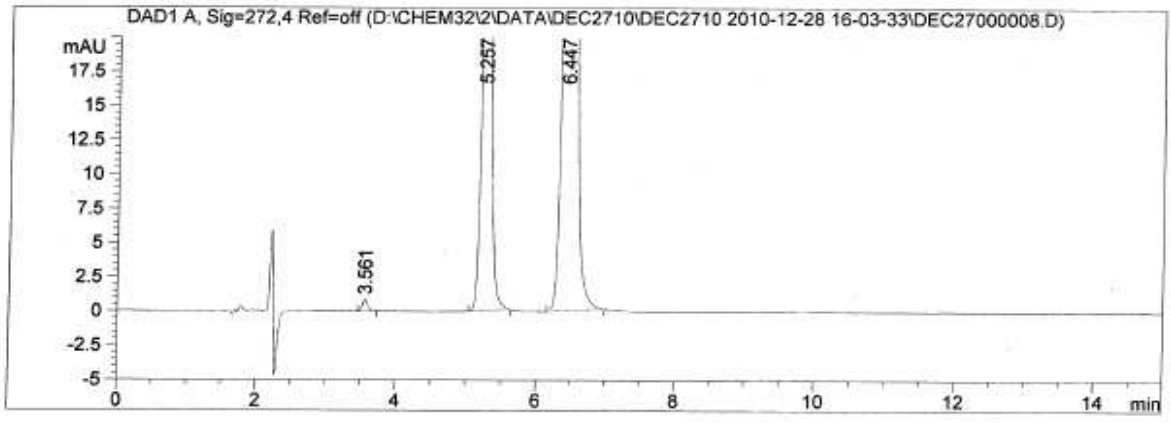


Figure 3 Chromatogram Of Acid Degradation (sample)

Stress Degradation studies:

Acid Degradation:

Sample Preparation: Twenty tablets were weighed, their mean weight was determined, and they were crushed in a mortar. An amount of powdered mass equivalent to Etofylline 100 mg and Theophylline equivalent to 30 mg weighed, add 20ml of 1N HCL, heat at 100°C for 3hr on

water-bath, after heating neutralize with the 20ml of 1N NaOH solution and make upto mark with water in 100ml volumetric flask. 5 mL aliquot from the stock solution of sample in 1N HCL was transferred in 50 mL volumetric flask, and the volume was made up to the mark with water. Blank solution is also treated with same procedure.

Alkali Degradation:

Sample Preparation: Twenty tablets were weighed, their mean weight was determined, and they were crushed in a mortar. An amount of powdered mass equivalent to Etofylline 100 mg and Theophylline equivalent to 30 mg weighed, add 20ml of 1N NaOH, heat at 100°C for 3hr on water-bath, after heating neutralize with the 20ml 1N HCL solution and make upto mark with water in 100ml volumetric flask. 5 mL aliquot from the stock solution of sample in 1N NaOH was transferred in 50 mL volumetric flask, and the volume was made up to the mark with water. Blank solution is also treated with same procedure.

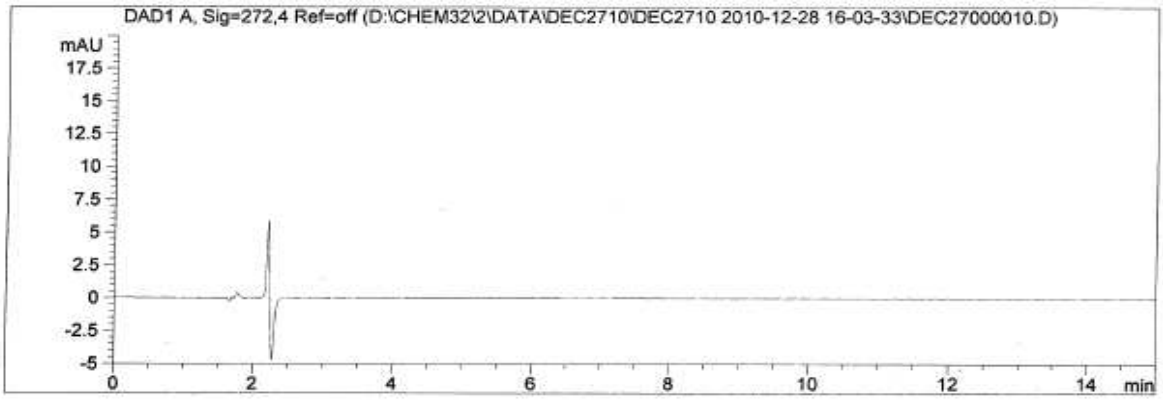


Figure 4 Chromatogram Of Alkali Degradation (blank)

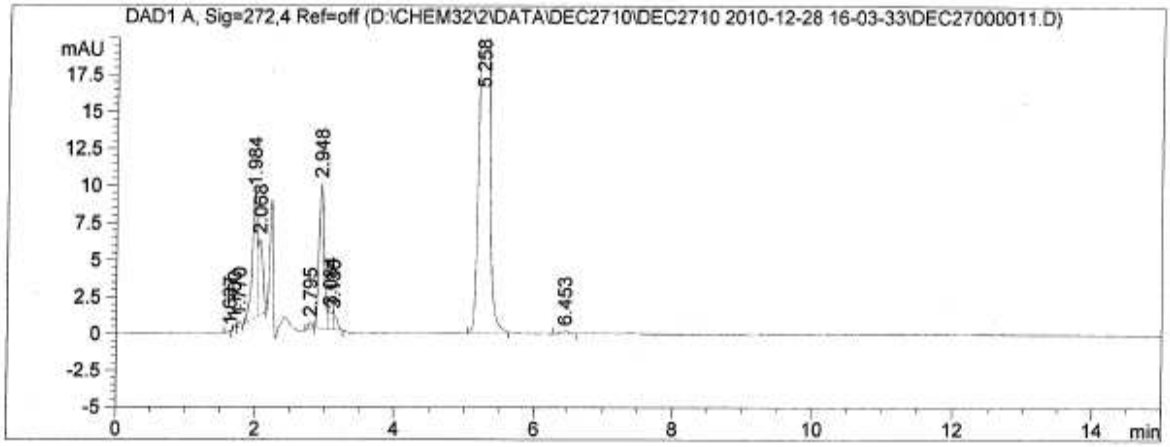


Figure 5 Chromatogram Of Alkali Degradation (sample)

Table 2 Result of Forced Degradation Study

	Purity Factor	
Stress Condition	Etofylline	theophylline
Acidic/1N HCl/100°C/3hr/solution	999.985	999.942
Alkaline/1N NaOH/100°C /3hr/solution	Degraded	999.975

Method Validation

Validation was carried out with respect to various parameters, as required under ICH guidelineQ2 (B).[9] The developed method validated with respect to parameters such as system suitability, precision, linearity, accuracy, robustness, ruggedness, LOD, LOQ and solution stability.

System suitability

System suitability was daily performed during entire validation of this method. The results of system suitability were presented (Table 3)

Table 3 System Suitability Parameter

	Theophylline		Etofylline	
	Area	**Avg. Area**	**Area**	**Avg. Area**
Standard	497.75		1252.99	
Standard	498.69		1255.18	
Standard	500.38		1258.84	
Standard	503.54		1268.48	
Standard	498.24		1255.26	
Standard	500.36	499.82	1260.88	1258.6
SD	**2.12**		**5.61**	
RSD	**0.42**		**0.45**	

Table 4 Precision

	Theophylline		Etofylline		Theophylline	Etofylline
	Area	**Avg. Area**	**Area**	**Avg. Area**	**% Assay**	**% Assay**
Standard	497.75		1252.99			
Standard	498.69		1255.18			
Standard	500.38		1258.84			
Standard	503.54		1268.48			
Standard	498.24		1255.26			
Standard	500.36	499.82	1260.88	1258.6		
SD	**2.12**		**5.61**			
RSD	**0.42**		**0.45**			
Sample Set-I	453.58		1233.38			
	452.16	452.87	1230.57	1231.98	90.36	97.14
Sample Set-II	452.22		1235.9			
	452.33	452.27	1236.21	1236.05	90.52	97.81
Sample Set-III	449.85		1224.1			
	450.19	450.02	1224.06	1224.08	89.79	96.86
Sample Set-IV	455.76		1238.37			
	455.48	455.62	1237.35	1237.86	90.82	97.85
Sample Set-V	450.53		1228.25			
	451.74	451.13	1232.94	1230.6	89.96	97.31
Sample Set-VI	450.49		1230.78			
	450.05	450.27	1230.05	1230.41	89.87	97.4
Average Assay					**90.22**	**97.39**
SD of Assay					**0.41**	**0.39**
RSD of Assay					**0.45**	**0.4**

Precision

The method precision was done by preparing six different sample preparations by one analyst under the same condition. The results were presented in Table 5. The results obtained were within 2% RSD in Table 4.

Linearity

The linearity was determined at 5 levels over the range of 50% to 150% of standard concentration. Etofylline and Theophylline standard stock solutions were prepared. The result obtained is in Table 5. r^2 value for Etofylline and Theophylline were 0.9998 and 0.9998 in figure 6 & 7.

Table 5 Linearity of Etofylline and Theophylline

	Theophylline		**Etofylline**	
	Area	**Avg. Area**	**Area**	**Avg. Area**
Standard	487.01		1182.12	
Standard	486.48		1180.64	
Standard	486.99		1183.56	
Standard	487.06		1182.75	
Standard	487.2		1182.38	
Standard	485.94	486.78	1180.29	1181.96
SD	**0.48**		**1.26**	
RSD	**0.1**		**0.11**	
Sample 50 %	210.03		576.64	
	209.74	209.89	576.16	576.40
Sample 75 %	315.26		865.46	
	316	315.63	866.84	866.15
Sample 100 %	419.31		1151.43	
	418.7	419	1149.29	1150.36
Sample 125 %	519.43		1426.27	
	517.88	518.66	1422.57	1424.42
Sample 150 %	628.04		1724.17	
	630.16	629.1	1729.54	1726.86

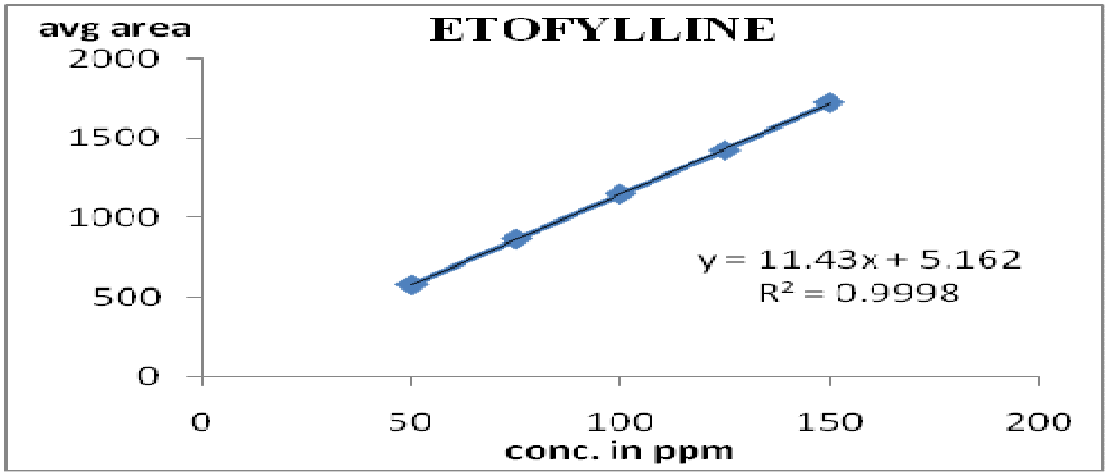


Figure 6 Linearity curve for Etofylline

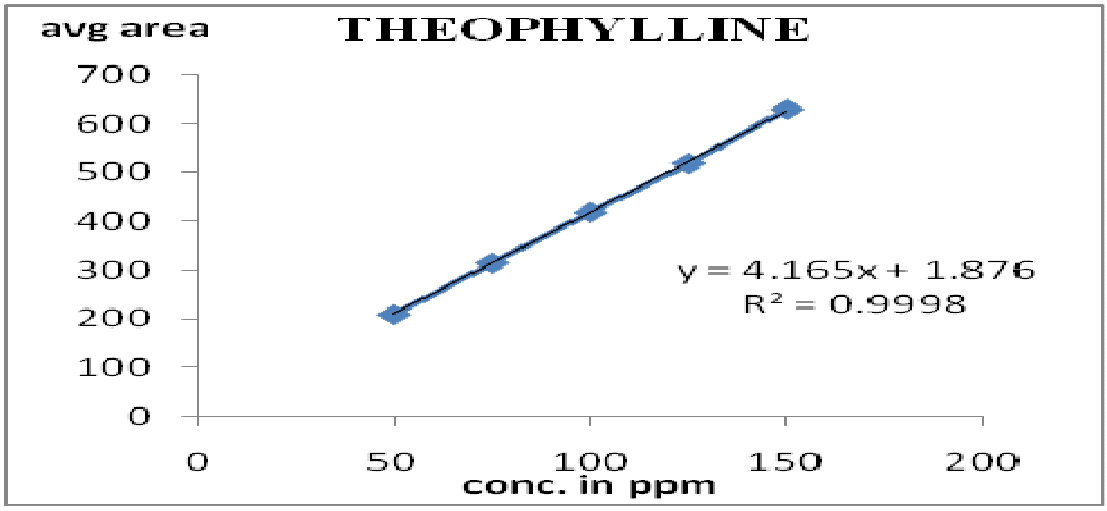


Figure 7 Linearity curve for Theophylline

Table 6 % Recovery

	Avg Area		Avg Assay		% Recovery	
	Theophylline	Etofylline	Theophylline	Etofylline	Theophylline	Etofylline
Sample 50%	209.89	576.4	43.52	49.01	100.18	100.21
Sample 75%	315.63	866.15	65.45	73.65	100.44	100.39
Sample 100%	419	1150.36	86.89	97.82	100	100
Sample 125%	518.66	1424.42	107.55	121.12	99.03	99.06
Sample 150%	629.1	1726.86	130.45	146.84	100.09	100.08

Table 7 Standard Condition For Robustness

	Theophylline		Etofylline		Theophylline	Etofylline
	Area	**Avg. Area**	**Area**	**Avg. Area**	**% Assay**	**% Assay**
Standard	473.23		1162.52			
Standard	473.15		1162.52			
Standard	473.08		1162.28			
Standard	472.62		1161.26			
Standard	472.42		1161.79			
Standard	472.39	472.82	1161.32	1161.95		
SD	0.38		0.57			
RSD	0.08		0.05			
Sample	425.71		1162.41			
	425.28	425.5	1161.71	1162.06	89.81	99.71
SD	0.3		0.5			
RSD	0.07		0.04			

Limit of Detection and Limit of Quantification

The limit of detection (LOD) and the limit of quantification (LOQ) of the drug were calculated using the following equations as per International Conference on Harmonization (ICH) guidelines (41). The LOD and LOQ for Etofylline are 0.3633 & 1.1010 and for Theophylline are 0.3805 & 1.1531.

LOD = 3.3 × σ/S
LOQ = 10 × σ/S
Where σ = standard deviation of the response
S= slope of the regression line

Table 8 Temperature Variation For Robustness (28˚C)

	Theophylline		Etofylline		Theophylline	Etofylline	% BIAS	
	Area	**Avg. Area**	**Area**	**Avg. Area**	**% Assay**	**% Assay**	Theophylline	Etofylline
Standard	473.33		1160.61					
Standard	474.36		1162.39					
Standard	473.11		1160.14					
Standard	473.35		1160.56					
Standard	473.56		1160.81					
Standard	472.49	473.37	1158.55	1160.51				
SD	0.61		1.23					
RSD	0.13		0.11					
Sample	425.66		1160.65					
	426	425.83	1160.67	1160.66	89.78	99.71	-0.04	0.00
SD	0.24		0.02					
RSD	0.06		0					

Table 9 Temperature Variation For Robustness (32˚C)

	Theophylline		Etofylline		Theophylline	Etofylline	% BIAS	
	Area	**Avg. Area**	**Area**	**Avg. Area**	**% Assay**	**% Assay**	Theophylline	Etofylline
Standard	473.16		1161.88					
Standard	473.16		1162.19					
Standard	473.2		1161.74					
Standard	472.9		1161.33					
Standard	473.54		1162.24					
Standard	472.86	473.14	1161.06	1161.74				
SD	0.24		0.47					
RSD	0.05		0.04					
Sample	426.97		1165.57					
	426.78	426.88	1165.13	1165.35	90.04	100.01	0.26	0.3
SD	0.13		0.31					
RSD	0.03		0.03					

Accuracy (% Recovery)

The difference between theoretical added amount and practically achieved amount is called accuracy of analytical method. Accuracy was determined at 5 different level 50%, 75%, 100%, 125% and 150% of the target concentration in duplicate. Result of accuracy data presented in Table 6.

Robustness

Robustness of the method was carried out by deliberately made small change in the flow rate, and organic phase ratio, column oven temperature. Results were presented in Table 7-13.

Table 10 Flow rate Variation For Robustness (0.9ml/min)

	Theophylline		Etofylline		Theophylline	Etofylline	% BIAS	
	Area	**Avg. Area**	**Area**	**Avg. Area**	**% Assay**	**% Assay**	Theophylline	Etofylline
Standard	525.3		1288.71					
Standard	525.92		1290.27					
Standard	525.24		1289.05					
Standard	525.22		1289.83					
Standard	526.81		1292.96					
Standard	525.74	525.71	1291.16	1290.33				
SD	0.61		1.56					
RSD	0.12		0.12					
Sample	474.34		1294.05					
	474.2	474.27	1294.38	1294.22	90.04	100	0.25	0.29
SD	0.1		0.23					
RSD	0.02		0.02					

Table 11 Flow rate Variation For Robustness (1.1ml/min)

	Theophylline		Etofylline		Theophylline	Etofylline	% BIAS	
	Area	**Avg. Area**	**Area**	**Avg. Area**	**% Assay**	**% Assay**	Theophylline	Etofylline
Standard	430.73		1060.94					
Standard	432.15		1064.28					
Standard	490.8		1059.96					
Standard	430.28		1059.62					
Standard	430.26		1059.13					
Standard	430.41	440.77	1060.27	1060.7				
SD	24.52		1.86					
RSD	5.56		0.18					
Sample	390.23		1063.24					
	391	390.61	1064.41	1063.83	88.44	99.99	-1.52	0.28
SD	0.55		0.82					
RSD	0.14		0.08					

Ruggedness

Ruggedness test was determined between two different analysts, instruments and Columns. The value of percentage RSD was below 2.0%, showed ruggedness of developed analytical method. The results were presented in Table 14.

Solution stability

The standard and sample solutions were found stable up to 24 hours at room temperature. After 3, 6, 9, 12, 15, 18, 21, 24 hours the solutions were analysed. No significant changes (<2%) were observed for the chromatographic responses for the solution analysed, relative to freshly prepared standard. Results related to solution stability are summarized in Table 15.

Table 12 Mobile Phase Composition Variation For Robustness (88:12)

	Theophylline		Etofylline		Theophylline	Etofylline	% BIAS	
	Area	**Avg. Area**	**Area**	**Avg. Area**	**% Assay**	**% Assay**	Theophylline	Etofylline
Standard	473.16		1163.71					
Standard	471.2		1159.09					
Standard	471.78		1160.22					
Standard	471.57		1159.53					
Standard	471.06		1159.09					
Standard	470.93	471.62	1158.97	1160.1				
SD	0.82		1.83					
RSD	0.17		0.16					
Sample	422.81		1155.49					
	422.8	422.81	1155.85	1155.67	89.47	99.32	-0.38	-0.39
SD	0		0.25					
RSD	0		0.02					

Table 13 Mobile Phase Composition Variation For Robustness (92:8)

	Theophylline		Etofylline		Theophylline	Etofylline	% BIAS	
	Area	**Avg. Area**	**Area**	**Avg. Area**	**% Assay**	**% Assay**	Theophylline	Etofylline
Standard	477.09		1170.08					
Standard	477.12		1170					
Standard	476.5		1168.51					
Standard	476.18		1168.24					
Standard	476.08		1167.34					
Standard	475.64	476.44	1166.04	1168.37				
SD	0.59		1.55					
RSD	0.12		0.13					
Sample	428.15		1166.37					
	427.7	427.92	1166.39	1166.38	89.64	99.53	-0.19	-0.18
SD	0.32		0.02					
RSD	0.07		0					

CONCLUSION

From the above study we can conclude that the Etofylline and Theophylline undergo degradation to different extent under different, above mentioned, stress conditions. In this study, the products formed after forced decomposition studies were resolved from the bulk drug response. From the peak purity profile studies, it was confirmed that the peak of the degradation product was not interfering with the peak of drugs. It confirms that peak for degradation product of drug can be resolved from the drug peak by this method. The developed method is simple, accurate, precise, and specific, economic. It is proposed simultaneous routine analysis of these drugs in presence of degradation products in stability study.

Table 14 Ruggedness

	Theophylline		Etofylline		Theophylline	Etofylline
	Area	**Avg. Area**	**Area**	**Avg. Area**	**% Assay**	**% Assay**
Standard	436		1083.36			
Standard	434.54		1081.01			
Standard	435.69		1083.31			
Standard	434.77		1081.61			
Standard	435.84		1083.68			
Standard	435.49	435.39	1082.7	1082.61		
SD	0.6		1.07			
RSD	0.14		0.1			
Sample	397.16		1096.74			
	397.58	397.37	1098.37	1097.56	90.75	101.04
SD	0.3		1.16			
RSD	0.07		0.11			
%BIAS					1.07	1.32

Table 15 Solution Stability Study

	THEOPHYLLINE	**ETOFYLLINE**	**%Difference**	
	Area	**Area**	**THEOPHYLLINE**	**ETOFYLLINE**
Standard - 0hour	457.45	1167.32	-	-
Standard - 3 hour	455.13	1162.65	-0.51	-0.4
Standard - 6 hour	455.43	1164.22	-0.44	-0.27
Standard - 9 hour	455.73	1165.85	-0.37	-0.13
Standard - 12 hour	456.38	1166.47	-0.23	-0.07
Standard - 15 hour	457.12	1167.4	-0.07	0.01
Standard - 18 hour	459.7	1164.52	0.49	-0.24
Standard - 21 hour	461.64	1148.1	0.92	-1.65
Standard - 24 hour	460.35	1146.78	0.64	-1.76

Acknowledgment:
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