WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 10, Issue 12, 1290-1302. Research Article ISSN 2277– 7105

ANALYTICAL METHOD DEVELOPMENT, VALIDATION AND STABILITY INDICATING ASSAY FOR ETOPHYLLINE AND THEOPHYLLINE BY RP-HPLC

Anshu Priya[1], Jai Shiv Chauhan[2], Gaurav Pratap Singh Jadaun[2], Meenakshi Dahiya[2], D. P. Pathak*[1]

[1]Delhi Institute of Pharmaceutical Sciences and Research, Mehrauli - Badarpur Rd, Sector 3, Pushp Vihar, New Delhi-110017, India.

[2]Indian Pharmacopoeia Commission, Block A, Sector 23, Raj Nagar, Ghaziabad, Uttar Pradesh-201002.

Article Received on
25 July 2021,

Revised on 15 August 2021,
Accepted on 05 Sept. 2021

DOI: 10.20959/wjpr202112-21673

***Corresponding Author**
Dr. D. P. Pathak
Delhi Institute of Pharmaceutical Sciences and Research, Mehrauli - Badarpur Rd, Sector 3, Pushp Vihar, New Delhi-110017, India.
Tel: +919818567796;
drdppathak@gmail.com

ABSTRACT

Etophylline and Theophylline Fixed dose combination (FDC) is a combination of two bronchodilator and belongs to methyl xanthine group of drugs. It is used for the treatment of asthma. A simple, accurate, precise, convenient, and validated reverse phase HPLC (RP-HPLC) technique has been developed for the assay determination of Etophylline and Theophylline in the parenteral formulation. The determination was carried out using a silica gel column packed with octlysilane, 250 mm × 4.6 mm, i.d., 5 µm particle size and an isocratic mode was adopted for the separation. The mobile phase used for the separation was a mixture of 0.01M Sodium acetate and Methanol (90:10), pH 3.5 and flow rate was 2ml per min. The wavelength chosen for the detection of the drug was 272 nm at ambient temperature and the retention time found was 7.31 min. and 8.33 min. for Theophylline and Etophylline respectively. Recovery values for Theophylline and Etophylline were 98.50-102.90%. The method adopted has shown prominent results as great peak separation, better resolution, linear curve, good % recovery and accurate % RSD values make this method ideal for the determination of Etophylline and Theophylline in the parenteral formulation.

KEYWORDS: Theophylline, Etophylline, RP-HPLC, method development, validation, stability.

1. INTRODUCTION

Etophylline: Etophylline[1] is a chemical derivative of Theophylline,[2] which belongs to the class Xanthine. It is indicated in asthma[3] chronic obstructive pulmonary disease (COPD),[4] chronic bronchitis and emphysema.[5] Etophylline or hydroxyethyl theophylline could be a bronchodilator and is widely used in treatment with theophylline. (1) **Physicochemical properties:** Etophylline is an almost white crystalline powder.[6] The chemical name is 7-(2-hydroxyethyl)-1, 3-dimethyl-3, 7dihydro-1H-purine-2, 6- Dione.[7]

Theophylline: Theophylline is a dimethylxanthine having two methyl groups present at 1 and 3 positions. Theophylline could be used to treat a respiratory disease like chronic obstructive pulmonary disease (COPD) and Asthma, the chemical name of theophylline is 1,3-dimethyl xanthine. It belongs to the xanthine family, which is naturally found in tea and cocoa. (5) **Physicochemical properties:** Theophylline is also a white crystalline powder. The chemical name is Dimethylxanthine. Theophylline is soluble in the water and five other organic solvents as methanol, ethanol, 1-propanol, ethyl acetate, and acetone at ambient temperature and atmospheric pressure. It was found that the solubility of theophylline increases with an increase in temperature.

Mechanism of action

Theophylline acts in the airways of patients where reversible obstruction takes place, two of them are; bronchodilation[8] and non-bronchodilator prophylactic effects. The mechanism of action of Theophylline is known with uncertainty; preclinical studies suggest that bronchodilation could be moderated by the inhibition of two isozymes of phosphodiesterase (PDE III and to a lesser extent, PDE IV), on the other hand, non-bronchodilator actions are probably moderated through one or more different molecular mechanisms, that do not involve inhibition of PDE III or antagonism of adenosine receptors.[2]

Side effects: Frequently occurring side effects related to Theophylline appears to be hypotension, tachycardia, a headache, and emesis and alterations in cerebral blood flow.

Etophylline: Etophylline[1] is the ethyl salt of Theophylline. It inhibits the phosphodiesterase enzyme which in turn degrades cyclic nucleotides inside the cell, and it results in cyclic AMP

accumulation in the cell. This cause bronchodilatation, cardiac stimulation and vasodilatation. This drug release calcium from the sarcoplasmic reticulum, especially in cardiac muscles and results in increased cardiac muscle contraction.[1] Adenosine receptors (adenosine acts as a local moderator in CNS & CVS and other organs responsible for contraction of smooth muscles, especially in bronchi, blood vessels, etc) are also blocked by the drug. This results bronchodilatation and vasodilatation.

Although the pharmacological actions of the **Etophylline** are generally considered as those of theophylline.[9] Unlike other xanthine derivatives, Etophylline has wide therapeutic window because it is not converted to theophylline in body and a combination of etophylline and theophylline[2] shows less frequent adverse side effects than an equivalent dose of theophylline alone.[10]

Side effects: Common side effects of Theophylline are upset stomach, Stomach pain, Diarrhoea, Headache, Restlessness, Insomnia, Irritability, Vomiting, Increased or rapid heart rate, Irregular heartbeat, Seizures, Skin rash

Analytical method development followed by method validation plays a vital role in drug discovery.[11] Many methods were discussed for the determination of etophylline and theophylline alone and in combination with other drugs, but there was a limited number of methods reported for both the drug in combination.[12] The main objective of this work was to develop and validate the RP-HPLC method.[13,14] with UV[15] detection for the determination of etophylline and theophylline in the parenteral dosage form.[10,16]

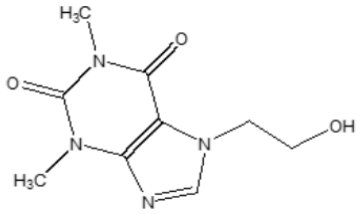

Fig. 1: Structure of etophylline.

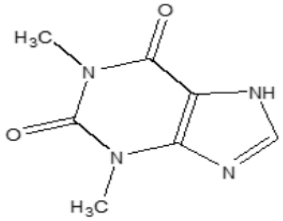

Fig. 2: Structure of theophylline.

2. MATERIALS AND METHODS

2.1 Chemicals and Solvents

The following chemicals and solvents are used in the study:

Table 1: Chemicals and Solvents.

S. no.	Chemical/solvent	Provider
1	Etophylline & theophylline iprs	Indian pharmacopoeia commission
2	Methanol	Rankem
3	Sodium acetate	Emplura
4	Ultra-high pure water	Miliq millipore (self-prepared)
5	Sample	Zydus cadila (batch no.: bgw1057)

2.2 Preparation of mobile phase

A degassed mixture of 0.01M Sodium Acetate and Methanol in the ratio of 90;10 v/v was prepared. The Buffer was filtered through 0.5 µ membrane filters. The mobile phase was running separately.

2.3 Standard preparation

Weigh 42.5 mg of Etophylline and 12.7 mg of Theophylline working standard and transferred into a 50 ml clean and dry graduated volumetric flask. Then volume makes up was done with HPLC grade water. It was further diluted by pipetting 2 ml of the above solution and transferred into a 20ml volumetric flask and then makeup with HPLC grade water. The final concentration of Etophylline (85ppm) & Theophylline (25ppm) was prepared.

2.4 Sample preparation

A 2ml of the sample from Deriphyllin Injection were taken and transferred accurately to a 100ml clean and dry volumetric flask and the volume was made with HPLC grade water. It was further diluted by pipetting out 5ml of the above solution and transferred into a 100ml volumetric flask and final volume make up was done by HPLC grade water. The final concentration of Etophylline (85ppm) & Theophylline (25ppm) was prepared.

2.5 Chromatographic conditions

The chromatographic conditions used for the validation of Etophylline and Theophylline are given in table 2:

Table 2: Chromatographic conditions.

HPLC SYSTEM	Thermo Scientific Dionex Ultimate 3000
Software Used	Chromeleon
Column	Waters Sunfire C8, 250mm×4.6mm, 5µm
Mobile Phase	90:10 v/v (Buffer: Methanol) pH 3.5±0.05
Flow rate	2ml/min.
Injection Volume	20 µl
Detector	UV
Wavelength	272nm
Run Time	10 min.
Column temperature	25 ºC

2.6 Assay procedure

A 20.0 µl of a placebo, standard preparation (six times) and sample preparation (two times) were separately injected into the chromatographic system. Then afterwards, the chromatograms and the peak responses of standard and sample preparations were observed. The chromatogram of standard preparation was recorded, and the peak responses were measured. The number of theoretical plates should not be less than 2000 & the tailing factor for the principal peak should not be more than 2.0. The % RSD (Relative Standard Deviation) should not be more than 2.0.

3. RESULT AND DISCUSSION

To predict the chromatographic behaviour, chemical structure and chemical properties play a very important role. In the present study, the best resolution was achieved using Thermo Scientific HPLC, consisting of Silica bonded, packed with octylsilane (250mm×4.6mm), 5 µm and mobile phase buffer and methanol 90:10 v/v. The broadening of peaks and long analysis duration occurs due to the lower percentage of methanol in the mobile phase while a higher percentage of methanol in the mobile phase resulted in the merging of peaks. The optimal retention time was 7.357 min and 8.384 min for Theophylline and Etophylline were achieved when the pH was 3.5. A slight change in the pH of the mobile phase had a great influence on the chromatographic data of these substances. Higher pH of the mobile phase resulted in the merging of the peak while lower pH results in an increase in the retention time.

3.1 Chromatogram of Etophylline & Theophylline

A typical chromatogram of Etophylline & Theophylline Injection is shown in Fig.3.

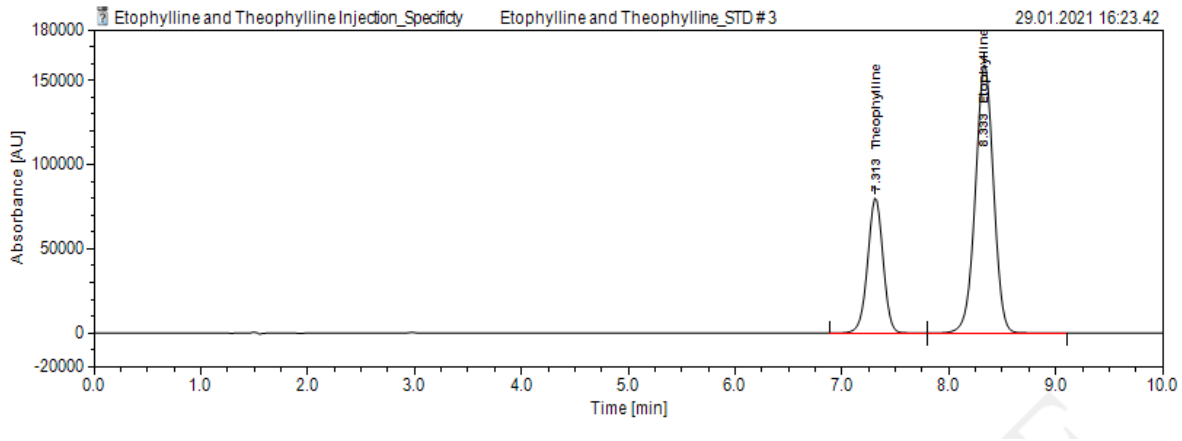

Fig. 3: Chromatogram of Etophylline & Theophylline.

The retention time for Theophylline and Etophylline are 7.357 min & 8.384min respectively. The variation in the retention times did not vary to any considerable degree during and in between analyses (% R.S.D. less than 2% for a retention time of each peak).

System suitability test is an integral part of the chromatographic system.[17] To establish the effectiveness of the method, a system suitability test is carried out on a freshly prepared standard stock solution of Etophylline & Theophylline. Table 3 shows the SST parameters.

Table 3: System suitability data.

Sample	RT (min)	Resolution	Plates	Tailing Factor	%RSD
Etophylline1	8.372	3.58	11535	0.97	0.04
Etophylline2	8.386	3.58	11592	0.98	
Etophylline3	8.379	3.58	11579	0.98	
Etophylline4	8.386	3.57	11476	0.99	
Etophylline5	8.399	3.56	11479	0.98	
Mean	**8.384**	-	-	-	0.05
Theophylline1	7.346	-	12471	0.98	
Theophylline2	7.359	-	12516	0.99	
Theophylline3	7.352	-	12512	0.99	
Theophylline4	7.359	-	12513	0.96	
Theophylline5	7.372	-	12439	0.98	
Mean	**7.357**	-	-	-	

3.2 Specificity

The solution of standard and sample was prepared as per the above-mentioned method and were injected into the chromatographic system. The chromatogram as well as retention time were identical for standard, and sample as shown in Table 4.

Table 4: Specificity of Etophylline and Theophylline.

S. no.	Peak	Retention Time (min.)	Area	Area (%)	Tailing factor	Resolution	Theoretical Plates
1	Etophylline	7.313	1883992.2836	70		3.60	11730
2	Theophylline	8.333	807469.0107	30		---	12619
Total			2691461.29	100			

3.3 Linearity

Linearity is established by measuring the response at various concentrations by a regression plot, typically by the method of least squares.[18] A series of solutions were prepared using working standards of Etophylline and Theophylline at concentration level from 40% to 160% of target concentrations.

Table 5: Linearity.

S. no.	Linearity conc. (%)	Etophylline	Theophylline
1.	40	778775.663	330708.215
2.	50	1035988.736	440867.791
3.	60	1215800.948	519059.928
4.	80	1525878.572	649689.007
5.	100	1934377.420	825994.367
6.	120	2387161.466	1020815.838
7.	140	2762493.323	1180004.012
8.	150	2915432.517	1242439.207
9.	160	3164756.727	1352403.908
Correlation Coefficient		0.998	0.998

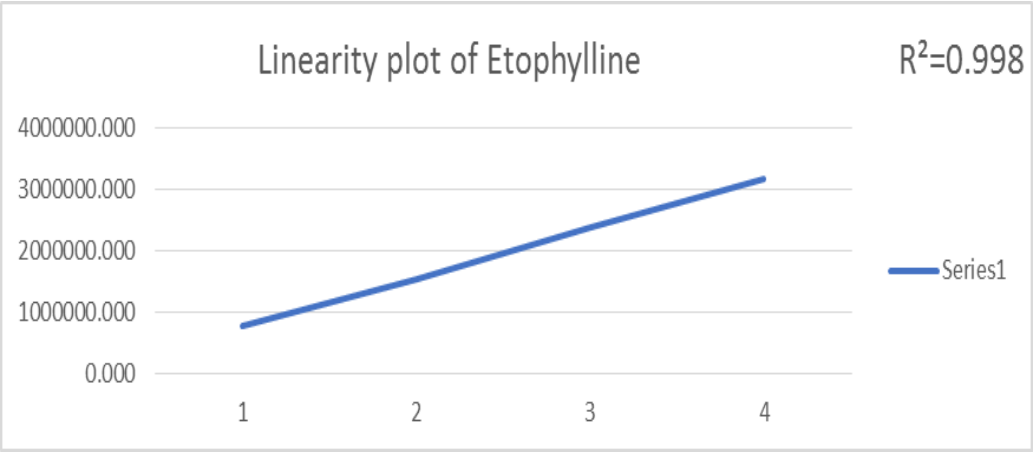

Fig. 4: Linearity plot of etophylline.

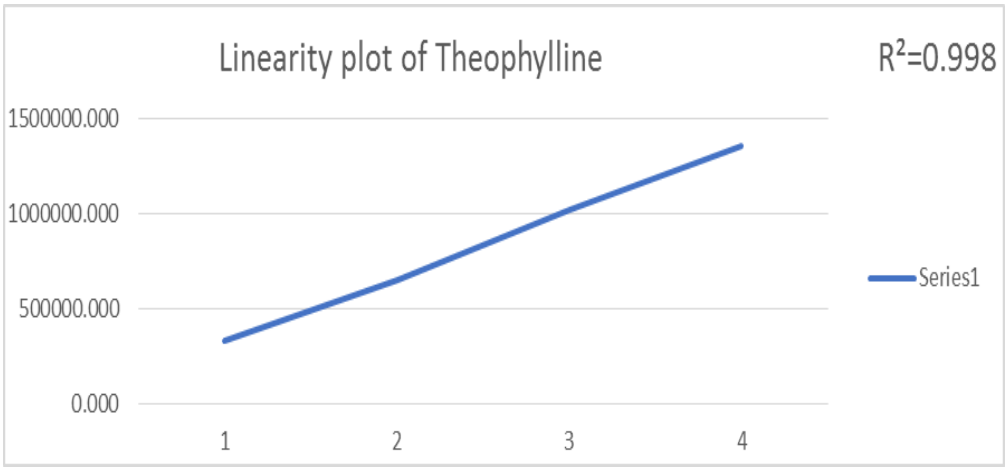

Fig. 5: Linearity plot of theophylline.

3.4 Precision

Precision may be considered at three levels: Method precision, intermediate precision, and System precision.[19]

Method precision was evaluated by the analysis of 6 samples, which were separately prepared from the same batch. During the analyses, separate sample solutions were injected, and the peak areas obtained were used to calculate the mean and % R.S.D. values. The result of method precision is shown in Table 3. The accepted criteria of R.S.D. less than 2% was met in all instances.

Table 6: Method precision.

S. no.	Sample	Peak area(mean)	% Assay	% RSD
1.	Etophylline	2036746.83	101.29	0.74
2.	Theophylline	745451.076	99.68	0.81

System precision

The system precision was investigated by injecting freshly prepares standard solution six times. The result obtained was all below the accepted minimum criteria of 2% R.S.D.

Table 7: System precision.

S. no.	Sample	Peak area(mean)	% Assay	%RSD
1.	Etophylline	2030600.18	100.28	0.63
2.	Theophylline	742421.29	98.78	0.66

Intermediate precision

Intermediate precision was studied and showed that the chromatographic patterns did not significantly change when different RP-HPLC system, analyst, the column was used. It was

seen that the percentage of R.S.D. was below 2%, which exhibits the ruggedness of a developed analytical method.

Table 8: Intermediate precision.

S. no.	Sample	Peak area(mean)	% Assay	% RSD
1.	Etophylline	188682.5260	100.21	1.24
2.	Theophylline	68565.9790	98.18	0.32

3.5 Accuracy

Method accuracy was studied by recovery investigation. Table 3 shows the results of the investigation. At the different concentration levels, the recovery values met the acceptance criteria of 100±2% for both the analytes. Along, these results provide the working range for the method.

Table 9: Accuracy.

S. no.	Sample	Concentration %	% Recovery(mean)
1.	Etophylline	80% Sample	98.27
		100% Sample	99.79
		120% Sample	101.96
2.	Theophylline	80% Sample	99.62
		100% Sample	101.25
		120% Sample	98.58

3.6 Robustness

The procedure to determine the robustness is to change the flow rate and wavelength. The contents of the analytes were not adversely affected by these small changes, it could be evident from the low relative standard deviation which indicates that the method is robust. However, a decrease in buffer volume resulted in the merging of peaks.

Note: Buffer volume cannot be decreased concerning methanol.

Table 10: Robustness.

S. no.	Parameters		Etophylline RSD %	Theophylline RSD%
1.	Flow rate	Increase	0.52	0.52
		Decrease	0.80	0.79
2.	Wavelength	Increase	1.05	1.08
		Decrease	1.14	1.17
3.	Buffer Increase		0.73	1.73

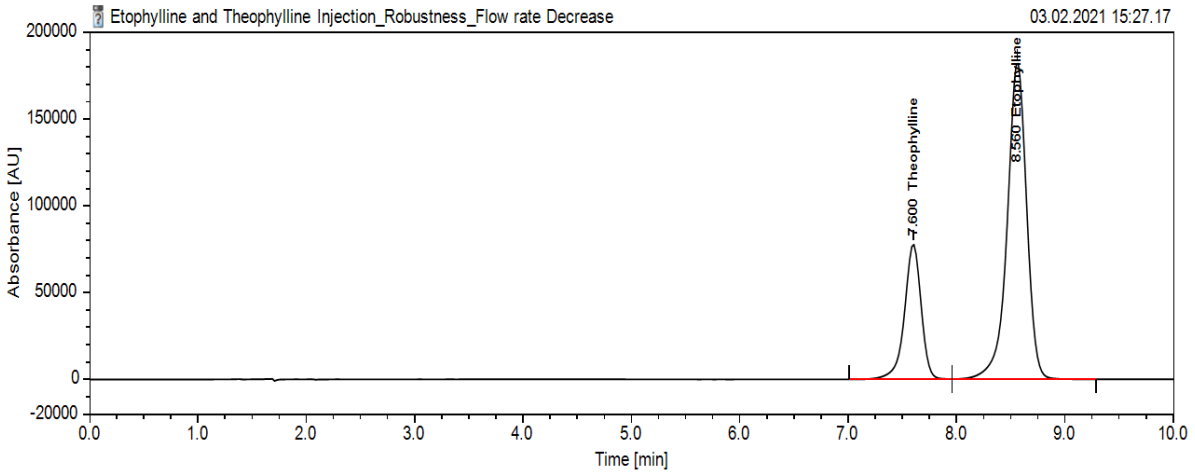

Fig. 6: A chromatogram of flow rate decrease.

3.7 Stability

It is important to have information about the stability of all the solution (20)(17) In this proposed study the stability of Etophylline and Theophylline in the working standard solution and sample preparation were analysed. Both analytes did not show evidence of significant degradation for at least 24 hrs when kept in medium (HPLC grade water). During this period, the results do not decrease below 98%.

The proposed method was found to be simple, specific and highly accurate, which require less time consumption for analysis, and this can be employed for routine analysis.

3.8 Forced degradation studies

The approach to analysing the stability of drug samples in pharmaceutical industries is provided by forced degradation studies.[21] The stability has an impact on purity, safety, and potency.[22] International Conference on Harmonisation (ICH) guidelines make it essential to organize the forced degradation studies. These data play a significant role which is required in the regulatory documentation.[21]

We have subjected the drug to different forced degradation studies like basic, acidic, oxidative, and thermal degradation. The results revealed that Etophylline and Theophylline drug was found to be stable at high temperature, unaffected by acidic hydrolysis, oxidation, alkaline hydrolysis, and resistance to photolysis.

3.9 Limit of Detection and Limit of quantification

The LOD and LOQ are calculated for the dosage form, and it is as follows:

Table 11: LOD and LOQ of Etophylline and Theophylline.

S. no.	Drug	Limit of detection	Limit of quantification
1	Etophylline	0.15	0.425
2	Theophylline	0.028	0.125

4. CONCLUSION

A simple, accurate, precise, convenient, and validated reverse phase HPLC method has been developed for the assay determination of Etophylline and Theophylline in the parenteral formulation. The method enables the quantification of Etophylline and Theophylline. The reagents used in the study are cost economic and the sample preparation was easy since both drugs were soluble in water. Along with this, both the compounds were eluted within 10 mins. The results obtained for all the parameters met the acceptance criteria and the proposed RP-HPLC method shows a sufficient resolution and hence can be used for the routine analysis of samples.

5. ACKNOWLEDGEMENTS

This study was supported by the Indian Pharmacopoeia Commission, Sector 23, Raj Nagar, Ghaziabad, Uttar Pradesh-201002.

Conflict of interest: All authors declared there was no conflict of interest involved**.**

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