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"DEVLOPMENT AND VALIDATION OF RP-HPLC METHOD FOR QUANTIFICATION OF THIOCOLCHICOSIDE AND DICLOFENAC DIETHYLAMINE IN PRESENCE OF ACTIVE EXCIPIENTS FROM THEIR COMBINED SEMISOLID TOPICAL PREPARATION"

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ABSTRACT:

A simple, accurate, precise, specific reverse phase high performace liquid chromatography method was developed and validated for quantification of Thiocolchicoside and Diclofenac Diethylamine in combined pharmaceutical formulation (gel). The approach was validated for Thiocolchicoside and Diclofenac Diethylamine using ICH recommendations over a range of 1-5 µg/mL and 9-45 µg/mL for Thiocolchicoside and Diclofenac Diethylamine respectively. An analytical column HYPERSIL ODS $C1_{8,}$ 250 mm*4.6 mm was utilized. At a flow rate of 1.0 ml/min, the mobile phase was Acetonitrile and 0.025M potassium hydrogen phosphate buffer in a (50:50 v/v) ratio. The elution was examined using a UV detector with a detection wavelength of 254 nm. The retention times for Thiocolchicoside and Diclofenac Diethylamine are 4.3 minutes and 9.8 minutes, respectively. The percentage recoveries for Thiocolchicoside and Diclofenac Diethylamine were 99.00-101.5 % and 99.11-100.22 %, respectively. The RSD values are not greater than 2%.

KEYWORDS: Thiocolchicoside, Diclofenac Diethylamine, RP-HPLC Method

INTRODUCTION

- 1.16% w/w for Diclofenac diethylamine and 0.125%w/w for thiocolchicoside. It is use for muscle relaxant activity. Without reducing muscular strength, it relieves muscle stiffness or spasm by acting on brain and spinal cord centres. This lessens muscular discomfort and enhances mobility. When in depth literature review was done following areas were addressed
- There are two-three RP-HPLC methods reported for estimation of both the drugs from the capsule formulation.
- In the above methods the retention time of Thiocolchicoside is around 1.5-2 minutes (Where there is expected column dead volume with 250 mm column), which is not considered as a good chromatographic separation.
- None of the above mentioned methods gives an idea regarding SSTs.
- None of the above mentioned methods gives an idea regarding the specificity of the method (Interference due to presence of excipient).
- Current formulation contains excipients which are it self active like methyl salicylate, and it is present in significant concentration in the gel formulation, so we need to develop a method which can specifically quantify Thiocholcicoside and diclofenac diethylamine in presence of salicylic acid and linseed oil.
- Hence, it was aimed at "Development and validation of RP-HPLC method for quantification of Thiocolchicoside and Diclofenac Diethylamine in presence of active excipients from their combined semisolid topical preparation."

Materials and Methods

- **Instrument specification**

Table 1 Instrument specification for High Performance LC

Make	Shimadzu
Model	LC 2010
Type	Binary Gradient
Detector	UV detector
Software	LC solution
Column	Hypersil ODS C_{18} (250*4.6 mm, 5 Micro-meter)
Pump	High Pressure Gradient (Reciprocating pump)

Table 1 Instrument specification for weighing balance

Make	Mettler Toledo
Sensitivity	0.1 milligram
Minimum weighing Capacity	1 milligram

Table 2 Instrument specification for melting point apparatus

Make	Gallenkamp
Design No.	889339

Table 3 Instrument Specification for UV double beam Spectrophotometer

Make	: Shimadzu
Model	: UV 1800
Type	: Double beam spectrophotometer
Detector	: Photodiode
Scanning Range	: 190 – 1100
Output	: %T & Absorbance
Software	: U.V. Probe 2.42

HPLC METHOD DEVELOPMENT

Selection of analytical/detection wavelength

A Working standard of DIC (18 µg.ml^{-1}) and THIO (2 µg.ml^{-1}) using methanol as a solvent, were scanned in UV 200-400 nm region and overlapped.

Sample preparation

	Preparation of solution
Master Stock Solution:	Accurately weighed THIO+DIC (10 mg+90 mg) dissolved in 100 ml methyl alcohol (100+900 µg.ml^{-1})
Standard Solution	Withdraw 100 µl from Master Stock Solution and make up to 10 ml with methyl alcohol THIO+DIC (1+9 µg.ml^{-1})

- **RESULTS AND DISCUSSION**

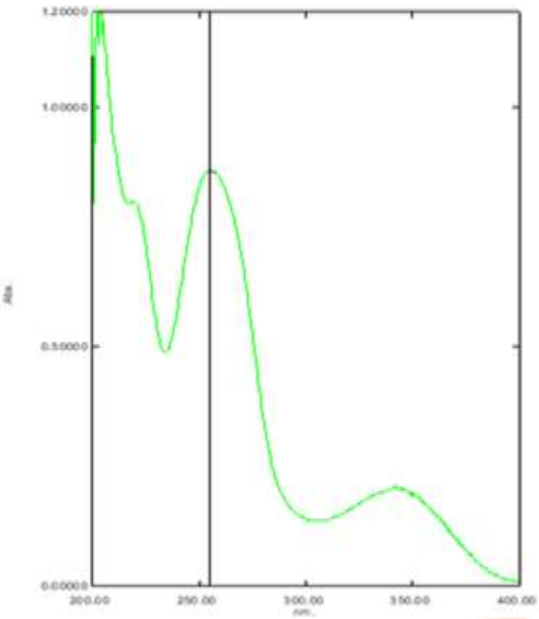
Identification of Thiocolchicoside

Identification by melting point

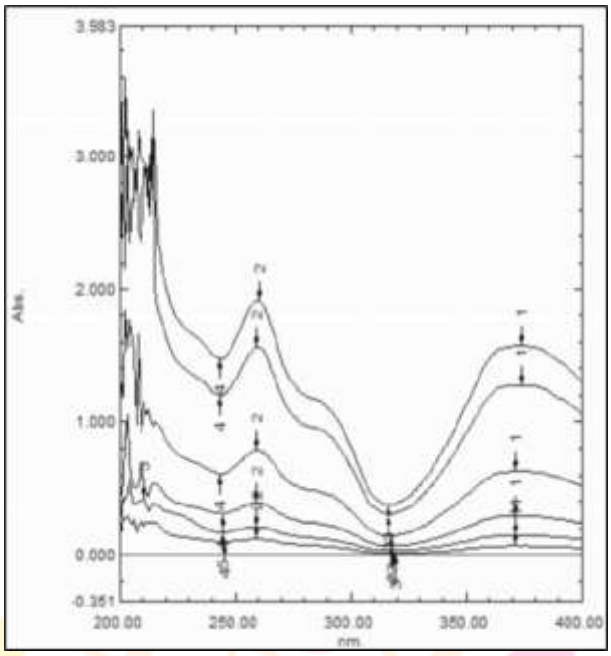
Table 2 Melting point data of Thiocolchicoside

Drug	**Reported Melting Point** [18]	**Observed Melting Point**
Thiocolchicoside	190-198 °C	193-199 °C

Identification by UV spectrophotometry



Recorded UV Spectra of Thiocolchicoside

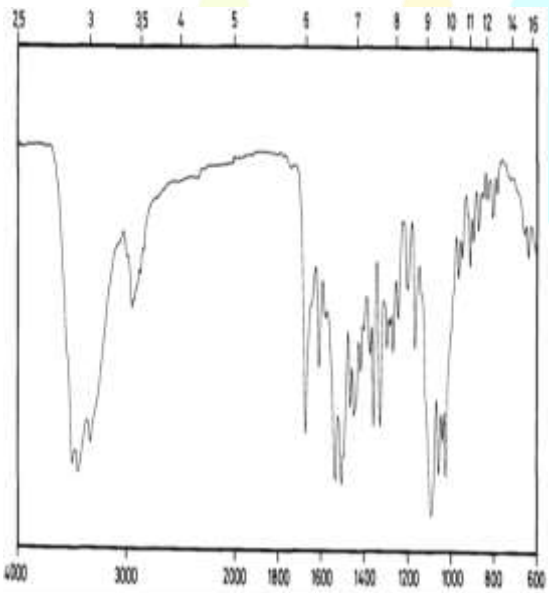


Reference UV Spectra of Thiocolchicoside [42]

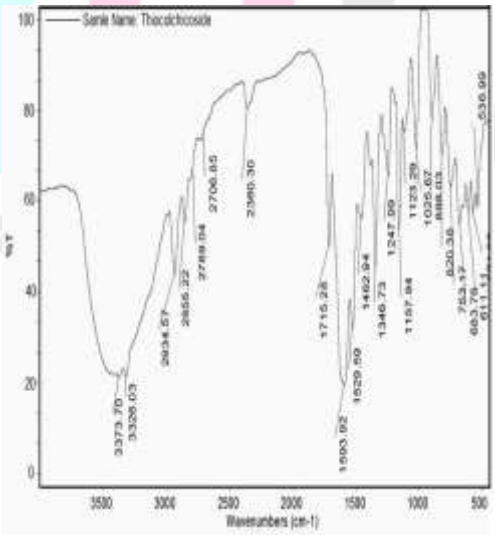
Comparison of reported and observed λ max

Drug	Solvent	Reported λ max	Observed λ max
Thiocolchicoside	0.1 M NaOH	259 nm	258 nm

Identification by IR Spectra



Reference IR Spectrum of Thiocolchicoside [43]



Recorded IR Spectrum of Thiocolchicoside

Interpretation of IR Spectrum of Thiocolchicoside

Type of Functional group/ Bond present	Reported frequency (cm^{-1})	Observed frequency (cm^{-1})
O-H Bending	1031	1025.67
O-H Stretch	3240	3326.03
N-H Stretch	3400-3200	3373.70
C=O Stretch	1680	1715.28
C-O Stretch	1251	1247.99
=C-H Stretch	3013	2934.57
C-H Bending	1470-1450	1462.94
C-S Stretch	700-600	683.76, 611.11

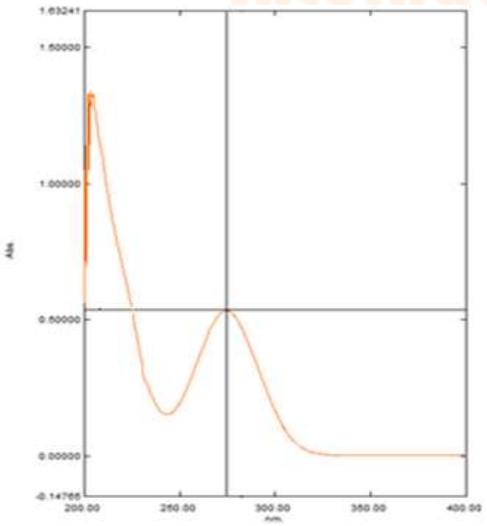
Identification of Diclofenac Diethylamine

Identification by melting point

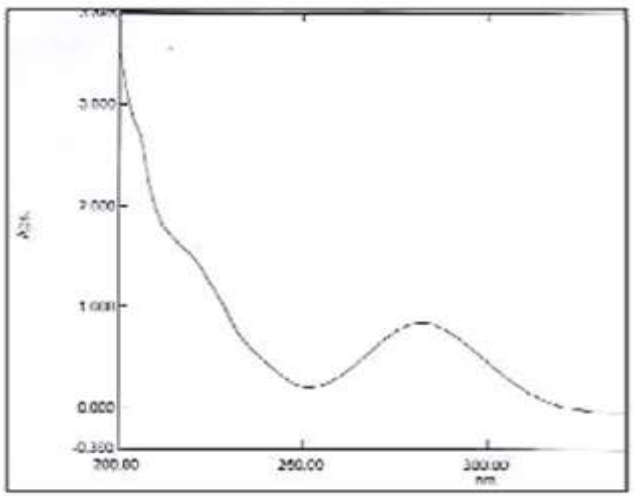
Table 3 Melting point data of Diclofenac Diethylamine

Drug	Reported Melting Point [22]	Observed Melting Point
Diclofenac Diethylamine	145-148°C	147-149°C

Identification by UV spectrophotometry



Recorded UV Spectra of Diclofenac Diethylamine

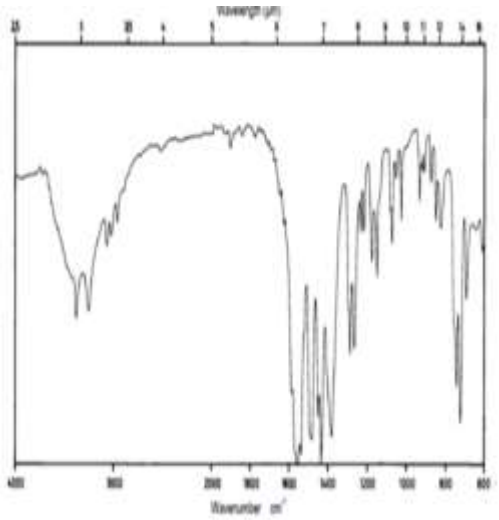


Reference UV Spectra of Diclofenac Diethylamine

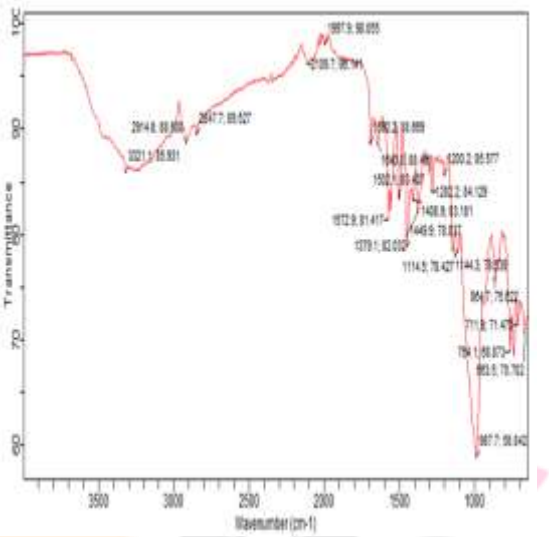
Comparison of reported and observed λ max

Drug	Solvent	Reported λ max	Observed λ max
Diclofenac Diethylamine	Methanol	275 nm	275 nm

Identification by IR Spectra



Reference IR Spectrum of Diclofenac Diethylamine [45]



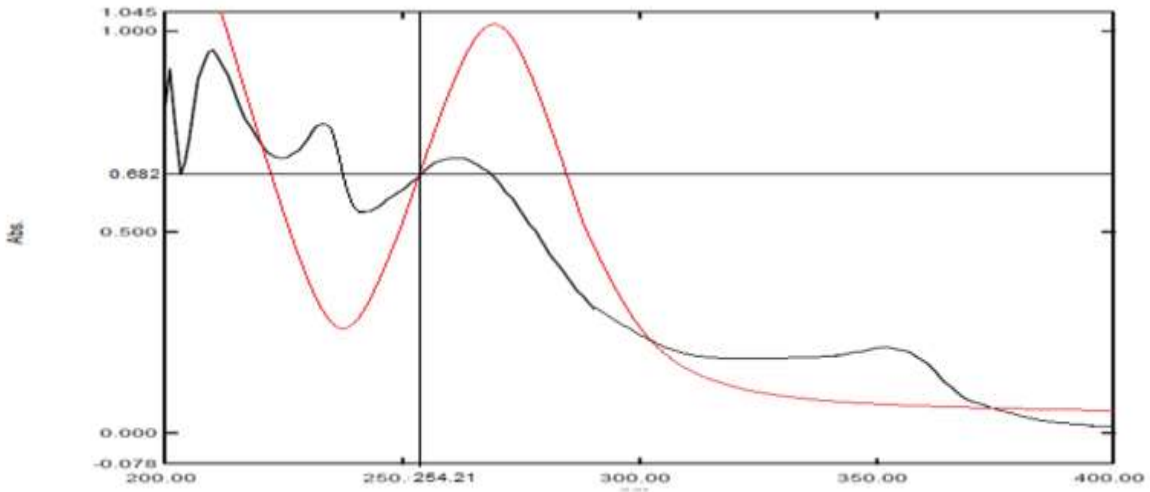
Recorded IR Spectrum of Diclofenac diethylamino

Interpretation of IR Spectrum of Diclofenac Diethylamine

Type of Functional group/ Bond present	Reported frequency (cm^{-1})	Observed frequency (cm^{-1})
=C-H stretch	3100-3000	3034
-C-H Stretch	2950-2800	2946, 2960
C=O stretch	1750-1735 (Ester)	1740, 1677
C-O stretch	1310-1250 (Ester)	1215
C-H bending	1450-1470	1474
C-N stretching	1350-1200	1375

High Performance Liquid Chromatography

Selection of Analytical/Detection wavelength



- Mandatory requirements for selection of analytical wavelength in HPLC with UV detection is that both the drugs should give adequate response at selected wavelength.
- The overlain spectra of individual component revealed that, at 254 nm both the components exhibited common absorption behaviour and hence it was selected for further method development purpose.

Optimization of Chromatographic Conditions

Trial 1 (As per reported method)

Column: Phenomenex C18 (250*4.6 mm, 5µm)

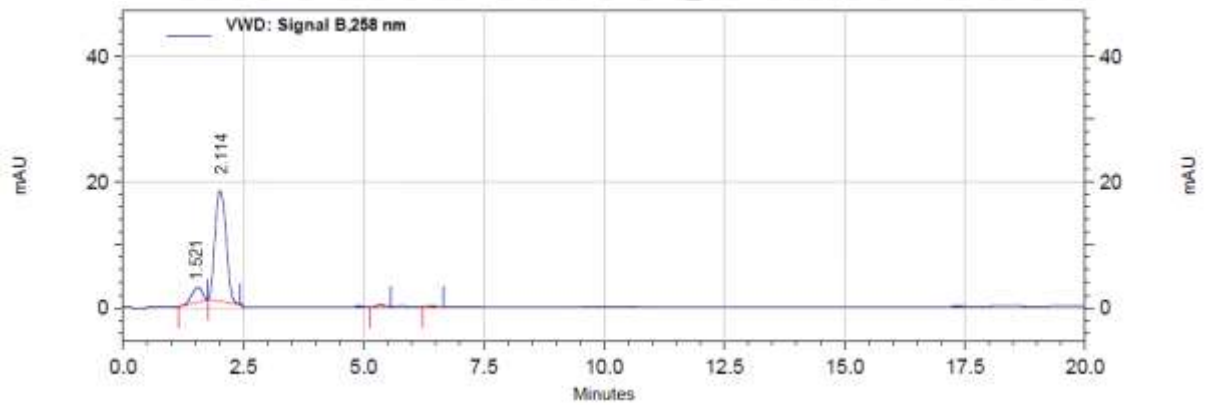
Mobile Phase: Acetonitrile: Water (70:30 v/v), pH-3

Detection: 258 nm

Flow rate: 1 ml/min

Run Time: 20 minutes

Observation: As mentioned in article, retention time of THIO was found near about 1.5 min. along with that, both drugs are not separated with adequate resolution



Trial 1: Chromatogram of THIO+DIC (1+9 µg.ml^{-1})

Trial 2

Column: Hypersil ODS C18 (250*4.6 mm, 5µm)

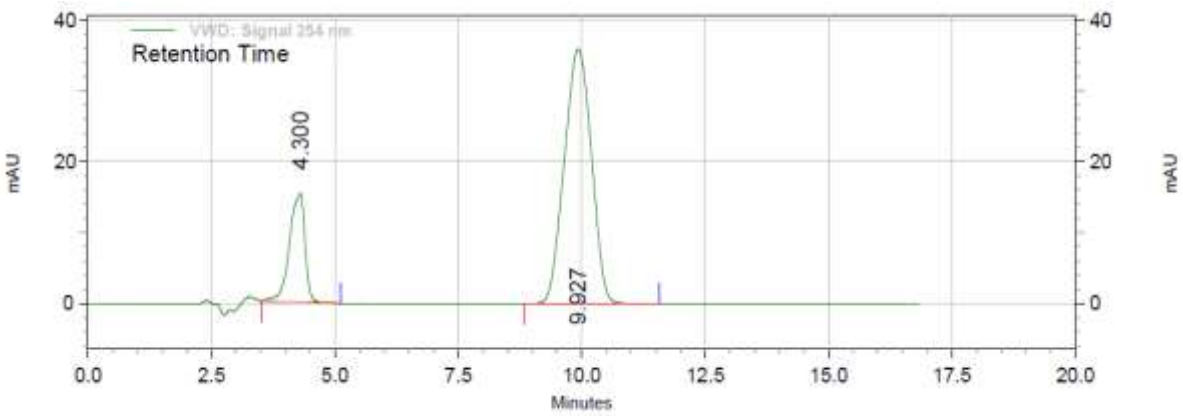
Mobile Phase: Acetonitrile: 0.025M potassium hydrogen phosphate buffer (50:50 v/v), pH-3

Detection: 254 nm

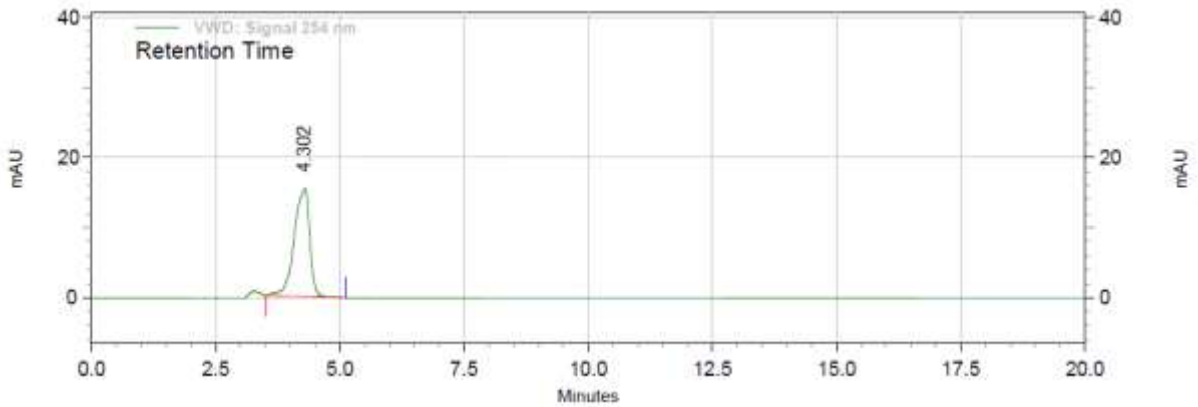
Flow rate: 1 ml/min

Run Time: 20 minutes

Observation: Separation with adequate resolution achieved



Trial 2: Chromatogram of THIO+DIC (1+9 µg.ml^{-1})



Chromatogram of THIO (1 µg.ml^{-1}) for peak identification

Optimized Chromatographic Condition

Table 4 Optimized Chromatographic Condition

Stationary Phase	HYPERSIL ODS $C1_{8,}$ 250 mm*4.6 mm
Mobile Phase	Acetonitrile: 0.025M potassium hydrogen phosphate buffer (50:50 v/v)
Detection wavelength	254 nm
Flow rate	1 ml/minute
Run Time	20 minutes
Retention Time	THIO: 4.3 min, DIC: 9.8 min

System Suitability Parameters

Table 6 System suitability parameter for THIO+DIC (1+9 µg.ml^{-1})

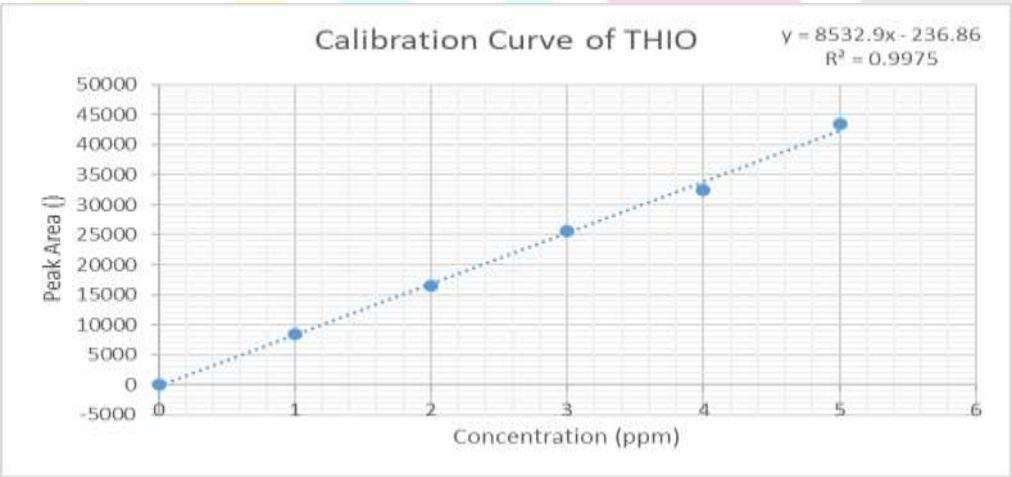
Parameter	THIO			DIC		
	Mean	± SD (n=3)	RSD	Mean	± SD (n=3)	RSD
Retention time (R_t)	4.31	0.00	0.05	9.91	0.01	0.09
Tailing Factor	0.96	0.02	2.08	1.19	0.02	1.28
Number of theoretical plates	9457.67	110.55	1.17	21469.33	81.65	0.38
Resolution (R_s)	6.26	0.08	1.25	6.26	0.08	1.25

Validation of developed RP-HPLC method for estimation of THIO and DIC

Linearity and Range

Table 7 Linearity data of THIO

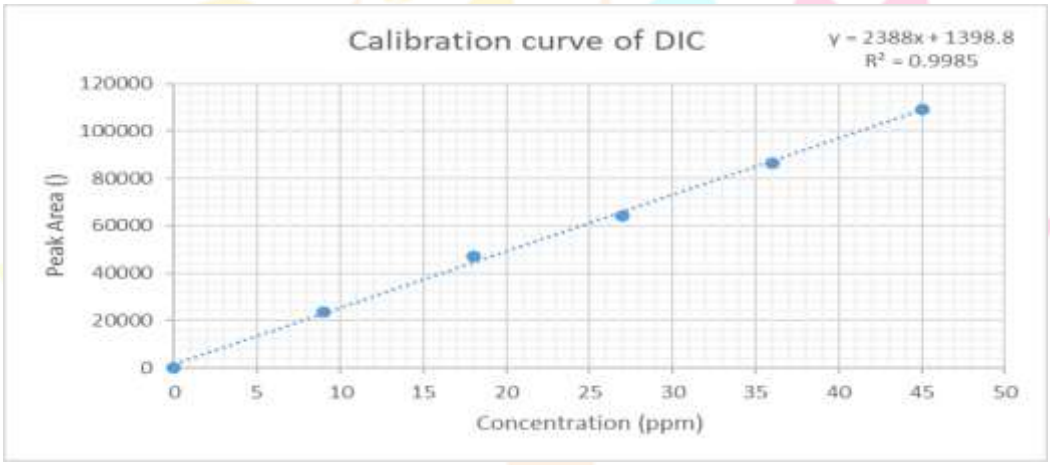
Sr. No.	Concentration (µg/ml)	Mean area (µV. s)	± SD (n=5)	RSD
1	1	8459.2	141.99	1.68
2	2	16549.6	210.50	1.27
3	3	25548.4	288.60	1.13
4	4	32522.8	332.35	1.02
5	5	43492.4	430.95	0.99



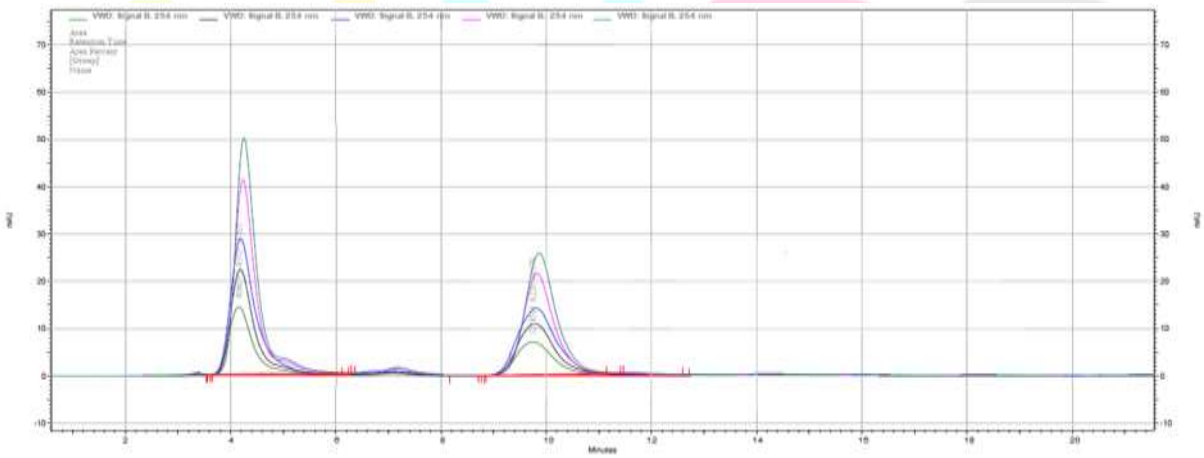
Calibration curve of THIO

Table 8 Linearity data of DIC

Sr. No.	Concentration (µg/ml)	Mean area (µV. s)	± SD (n=5)	RSD
1	9	23727.6	395.63	1.67
2	18	46920.8	535.47	1.14
3	27	64274.8	714.34	1.11
4	36	86602	786.32	0.91
5	45	109249.6	684.68	0.63



Calibration curve of DIC



Overlain Chromatogram for linearity

Conclusion:

As per ICH guidelines the value of R^2 should be greater than 0.995, and observed R^2 for given concentration range for THIO and DIC is 0.9975 and 0.9985 respectively.

Hence, we can say that developed method is linear over the range of 1-5 µg/mL and 9-45 µg/mL for THIO and DIC, respectively.

Repeatability

Table 9 Repeatability data of THIO

Sr. No.	Concentration (µg/mL)				
	1	2	3	4	5
1.	8325	16563	25413	32784	43215
2.	8465	16322	25964	32158	44129
3.	8311	16874	25316	32651	43065
4.	8563	16411	25733	32176	43332
5.	8632	16578	25316	32845	43721
MEAN	8459.2	16549.6	25548.4	32522.8	43492.4
± SD (n=5)	141.99	210.50	288.60	332.35	430.95
RSD	1.68	1.27	1.13	1.02	0.99

Table 10 Repeatability data of DIC

Conclusion:

Sr. No.	Concentration (µg/mL)				
	9	18	27	36	45
1.	23557	46231	64634	86118	109847
2.	23125	46842	64014	87995	108634
3.	23985	46845	64832	86423	109914
4.	24117	47732	64759	86239	108432
5.	23854	46954	63135	86235	109421
MEAN	23727.6	46920.8	64274.8	86602	109249.6
± SD (n=5)	395.63	535.47	714.34	786.32	684.68
RSD	1.67	1.14	1.11	0.91	0.63

As per ICH guidelines the value of RSD should be less than 2, and observed RSD is less than 2 for all concentrations of THIO and DIC.

Hence, we can say that developed method is repeatable over the range of 1-5 µg/mL and 9-45 µg/mL for THIO and DIC, respectively

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limit of Detection (LOD):

THIO	DIC
LOD = 3.3 x (σ/S) = 3.3 x (78.33/ 8532.9) = 0.0302 µg/mL	LOD = 3.3 x (σ/S) = 3.3 x (335.47 / 2388) = 0.463 µg/mL

Limit of Quantification (LOQ):

THIO	DIC
LOQ = 10 x (σ/S) = 10 x (78.33/ 8532.9) = 0.091 µg/mL	LOQ = 10 x (σ/S) = 10 x (335.47 / 2388) = 1.404 µg/mL

Accuracy

Table 11 Data for accuracy

Accuracy						
	Thio					
	50%		100%		150%	
	Amount of drug recovered	% Recovery	Amount of drug recovered	% Recovery	Amount of drug recovered	% Recovery
	0.99	99.00	1.98	99.00	3.04	101.33
	0.98	98.00	2.02	101.00	2.98	99.33
	1.01	101.00	2.03	101.50	2.97	99.00
Mean	0.99	99.33	2.01	100.50	3.00	99.89
SD	0.02	1.53	0.03	1.32	0.04	1.26
	Dic					
	50%		100%		150%	
	Amount of drug recovered	% Recovery	Amount of drug recovered	% Recovery	Amount of drug recovered	% Recovery
	8.89	98.78	18.04	100.22	26.82	99.33
	8.92	99.11	17.92	99.56	26.75	99.07

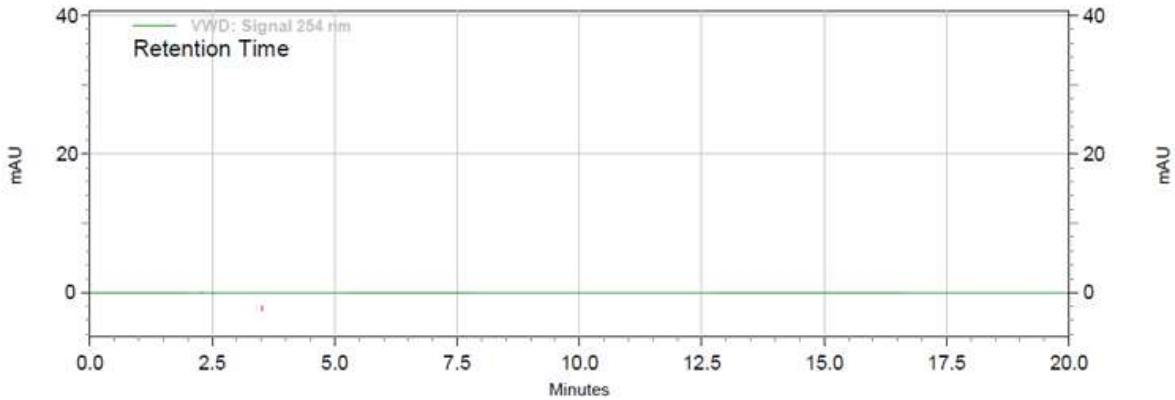
	8.85	98.33	17.84	99.11	26.84	99.41
Mean	8.89	98.74	17.93	99.63	26.80	99.27
SD	0.04	0.39	0.10	0.56	0.05	0.18

Robustness

Parameter	**Level of Change**	**Effect on assay volume**			
		THIO		**DIC**	
		Assay ± SD	**RSD**	**Assay ± SD**	**RSD**
Flowrate	0.9 mL/min	98.52 ± 0.39	0.39	99.75 ± 0.11	0.11
	1.1 mL/min	98.48 ± 0.23	0.23	100.46 ± 0.53	0.53
Mobile Phase Composition	52:48	99.26 ± 0.56	0.56	100.66 ± 0.33	0.33
	48:52	99.08 ± 0.48	0.49	98.41 ± 0.38	0.38

(n = 3 determinations)

Specificity



Chromatogram for Specificity

Specificity of the method was adjudged by injecting the mobile phase in optimized chromatographic condition, it was observed that no interference observed from mobile phase

Assay

Drug	Amount taken (µg/mL)	Amount found (µg/mL)	% Assay
THIO	1	0.99 ± 0.02	99.33 ± 1.53
DIC	9	8.87 ± 0.04	98.52 ± 0.45

(n = 3 determinations

Summary and Conclusion

Table 12 Optimized Chromatographic Condition	
Stationary Phase	HYPERSIL ODS $C1_{8,}$ 250 mm*4.6 mm
Mobile Phase	Acetonitrile: 0.025M potassium hydrogen phosphate buffer (50:50 v/v)
Detection wavelength	254 nm
Flow rate	1 ml/minute
Run Time	20 minutes
Retention Time	THIO: 4.3 min, DIC: 9.8 min

Table 4 Validation parameters				
Parameter	**Limit**	**Result**		**Conclusion**
		THIO	**DIC**	
Linearity and Range	$R^2 > 0.995$	0.9975 (1-5 µg/mL)	0.9985 (9-45 µg/mL)	Method was linear
Repeatability	RSD < 2	0.99-1.68	0.63-1.67	Method was repeatable
LOD	-	0.0302 µg/mL	0.463 µg/mL	-
LOQ	-	0.091 µg/mL	1.404 µg/mL	-
Intraday Precision	RSD < 2	0.43-0.81	0.62-0.96	Method was precise
Inter-Day Precision	RSD < 2	0.62-0.94	0.67-1.09	Method was precise
% Recovery	98 - 102 %	99.00-101.5 %	99.11-100.22 %	Method was accurate

Robustness	RSD < 2	0.23-0.56	0.11-0.53	Method was robust
Assay	-	99.33 ± 1.53	98.52 0.45	-

Thus, we found that method was comply with all the validation parameters according to ICH Q2R1 guideline.

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