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Statistical Evaluation of NP-HPTLC and RP-HPTLC for Estimation of Triflusal in Bulk and in Capsule Formulation

KEYWORDS: NP-HPTLC, RP-HPTLC, Triflusal, Validation, ANOVA, Student's t- test.

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ABSTRACT *A simple, rapid, selective and sensitive NP- HPTLC and RP-HPTLC/ densitometry method was developed and validated for the estimation of triflusal. For normal phase Toluene: Methanol: Triethylamine (8: 2: 0.5 v/v/v) and methanol: water: triethylamine (6:4:0.5 v/v) as a mobile phase in normal and reverse phase respectively. Both analyses were scanned with a camag scanner-3 at 314 nm. In normal phase and reverse phase Rf values 0.40 ± 0.02 and 058 ± 0.02 respectively. Linearity was studied in the concentration range of 1000 to 6000 ng band-1 for normal and 10000 to 35000 for reverse phase with correlation coefficient 0.999 for both methods. The result obtained shown that the method best fits for estimation of drug in capsule formulation and thus can be used for its routine analysis. The newly developed method was validated according to the ICH guidelines with respect to specificity, linearity, accuracy, precision and robustness. The analysis of variance (ANOVA) and Student's t-test were applied to compare and correlate the results of Triflusal determination in dosage form by means of normal and reverse phase thin layer liquid chromatographic method.*

Introduction

Triflusal (TRI) Fig 1, a benzoic acid derivative, is chemically 2-(acetyloxy)-4-(trifluoromethyl) benzoic acid, having molecular formula $C_{10}H_7F_3O_4$, molecular weight 248.155 gm/mol and melting point 118°c [1].

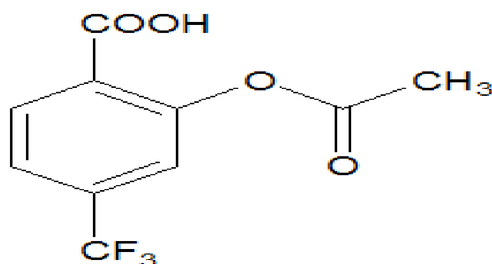

Figure 1: Chemical structure of Triflusal

The estimation of TRI is vital as the drug has multiple modes of action that added value in its definitive pharmacological effect. Apart from inhibition of prostaglandin synthesis as it resembles with that of aspirin, the drug also shows preservation of prostacyclin, blockage of phosphodiesterase thereby increasing cAMP concentration. It also acts as an antiplatelet agent that involves blockage of cyclooxygenase inhibiting thromboxane A2 [2].

The detail literature survey revealed few analytical methods reported for evaluation TRI such as HPLC [3-5]. HPLC with automated column switching system [6], spectroscopic and chromatographic characterization using supercritical impregnation technologies [7].

Promoted by above findings and the ultimate therapeutic utility of TRI, previously we have estimated the drug by simple UV spectrophotometric and RP- HPLC method [8]. In continuation to that, in the present work the estimation of TRI was done by more sophisticated technique i.e. using normal and reverse-phase high-performance thin layer liquid chromatographic method. With the objective of reducing analysis time and maintaining good efficiency, there has been substantial focus on high-speed chromatographic separations. Methods proposed for analytical purposes must be as economical as possible, to enable their use in routine quality control. Many spectroscopic methods have been proposed for analysis of similar drug but for the first time we are describing simple, sensitive, accurate, precise, rapid and economic chromatographic method for TRI in capsule dosage form. Our objective in the present investigation is to develop and validate an NP- HPTLC and RP-HPTLC method for estimation of triflusal (TRI) and their analysis in capsule.

Experimental

Chemicals & Reagents

Pure drug sample of TRI was obtained from Glenmark Pharmaceuticals Ltd., Nasik (India), HPLC grade acetonitrile and methanol was obtained from Merck India Ltd., All the solvents of HPLC grade used throughout the study. All the solvents and solutions were filtered through a membrane filter (Millipore Millex® FH, filter units, Durapore-PVDF, Polyethylene, 0.45 µm pore size). Drug formulation (capsule) Grendis® with label claim 300mg is used for estimation.

Instrumentation and chromatographic conditions

In normal phase, chromatography was performed on 20 cm ×10 cm aluminum-backed TLC plates coated with 0.20 mm layers of silica gel 60 F254S (Merck, Darmstadt, Germany, supplied by Merck India, Mumbai, India) while in reverse phase, 20 cm ×10 cm aluminum-backed RP-TLC plates coated with 200-µm layers of silica gel 60 RP-18 F254S were used. Before chromatography the plates were prewashed with methanol and activated at 105°C for 5 min in oven. The samples were applied as 6 mm wide bands with the help of Linomat 5 sample applicator (Muttenz, Switzerland) fitted with a 100-µL sample syringe (Hamilton, Bonaduz, Switzerland). The plate was developed in a pre-saturated Camag twin trough glass chamber (20 cm × 10 cm). In normal and reverse, **Toluene: Methanol: Triethylamine (8: 2: 0.5 v/v/v) and methanol: water: triethylamine (6:4:0.5 v/v)** respectively were used as mobile phases and for optimized chamber saturation time was 15 min and 30 min, respectively. The plates were developed to a distance of 8.0 cm and scanned densitometrically using Camag TLC Scanner 3 equipped with win CATS software version 1.3.0 at 254 nm for both method. The source of radiation utilized was deuterium lamp emitting

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a continuous UV spectrum between 200- 400 nm. Evaluation was performed using peak area with linear regression.

Sample preparation

Stock solutions (1000 µg mL-1) of TRI were prepared in HPLC grade methanol. The standard was prepared by progressive dilution of the stock solution.

Optimization of HPTLC method

a) Normal phase

Optimization of mobile phase was done with a view to separate both these drugs. Various proportions of toluene and methanol were tried as mobile phase; 8:2 v/v proportion shows tailing. Therefore, to whip this problem triethylamine was added that improved the spot characteristics and finally, toluene: methanol: triethylamine (8: 2: 0.5 v/v/v) showed well defined and resolved peaks when the chamber was saturated with mobile phase for 15 min at room temperature and no tailing was observed when plate was scanned at 314 nm. The R_f of TRI was found to be 0.40 ± 0.02. (Figure 2a)

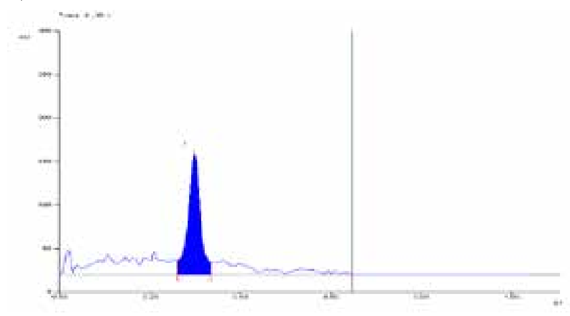

Figure 2 (a) Densitogram of standard TRI showing R_f 0.40

b) Reverse phase

Firstly, single solvents were selected on the basis of their polarity to separate the spots. Then the mixtures of solvents are used for optimization of TRI. The spots were developed in mixtures of methanol and water in the ratio of 3:2 v/v. The R_f value obtained was good but slight tailing was observed. Hence, to reduce the tailing, triethylamine was added in the solvent system. Thus, the final mobile phase consisted of methanol: water: triethylamine in the ratio (6:4:0.5 v/v). The chamber saturation time was 30 min. The R_f for TRI was found to be 0.58 ± 0.02. (Figure2b)

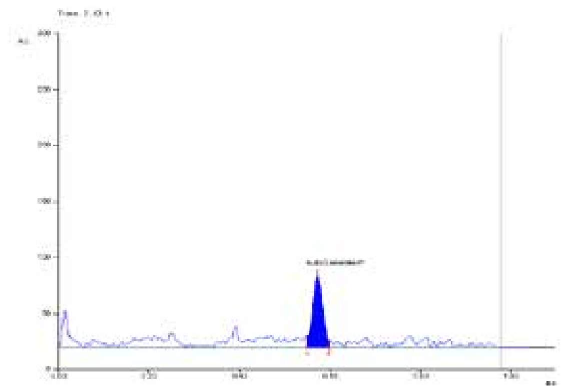

Figure 2 (b) Densitogram of standard TRI showing R_f 0.58

Validation

The developed method was validated as per ICH guidelines in terms of its linearity, accuracy, Limit of detection (LOD), Limit of quantification (LOQ), specificity, intra-day and inter-day precision and repeatability of measurement [9].

Linearity

From the stock standard solution, 1- 6 µL was applied on TLC plate to obtain series of concentration 1000 - 6000 ng band^{-1} of TRI for normal phase and 10- 35 µL was applied on RP-TLC plate to obtain series of concentration 10000- 35000 ng band^{-1} of TRI for reverse phase. The plates were developed and scanned as described. Each standard in six replicates was analyzed and peak areas were recorded calibration curves of TRI were plotted separately as peak area vs. respective concentration for both methods. The drugs showed good linearity in the tested range. The regression co-efficient (r^2) values for TRI were found to be 0.999 in both normal and reverse phase Table 1.

Table 1 Linearity of TRI for proposed method (n=6)

Parameters	Normal	Reverse
Linearity range (ng band^{-1})	1000 – 6000	10000 – 35000
Slope	0.901	0.205
Intercept	341	581.9
Correlation Coefficient (r^2)	0.999	0.999

Precision

Quantitative estimation of TRI was performed in order to validate and prove the applicability of the method; three series (2000, 3000, 4000 ng band-1 and 15000, 20000, 25000 ng band-1) were prepared for normal and reverse phase respectively Table 2.

Table 2 Precision of TRI for proposed method (n=3)

Methods	Conc ng band^{-1}	Intra-day precision Mean ± S.D.	% RSD [n = 3]	Inter-day precision Mean ± S.D.	% RSD [n = 3]
Normal phase	1500	2150 ± 13.11	0.60	2165 ± 8.62	0.39
	2000	3040 ± 17.43	0.57	3051 ± 24.94	0.81
	2500	3942 ± 27.66	0.70	3962 ± 16.82	0.42
Reverse Phase	15000	2482 ± 39.00	1.57	2499 ± 46.49	1.86
	20000	3539 ± 43.13	1.21	3550 ± 59.75	1.68
	25000	4523 ± 73.77	1.63	4553 ± 63.66	1.39

Accuracy

The accuracy of the experiment was established by spiking pre-analyzed sample with known amounts at three different concentration levels i.e. 80, 100 and 120 % of the drug in the capsules. The spiked samples were then analyzed for three times and the percentage recoveries were calculated by using formula.

$$\% \text{Recovery} = \frac{\text{Observed amount of compound in Sample}}{\text{Amount of all compound present in Sample}} \times 100$$

The mean recovery is found within acceptable limits indicates that the method is accurate for both phases which are tabulated in **Table 3.**

Table 3 Recovery studies

Methods	Initial amount (ng per band)	Amount added (%)	% recovery	%RSD [n=3]
Normal phase	2000	80	101.68	0.51
	2000	100	99.82	0.20
	2000	120	100.39	0.42
Reverse phase	20000	80	98.40	1.46
	20000	100	100.76	1.21
	20000	120	101.21	0.72

Analysis of the capsule dosage form

Twenty capsules (Grendis® 300 mg) were weighed accurately and crushed to form fine powder. Powder weight equivalent

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to 100 mg of TRI were dissolved in 100 mL volumetric flask with methanol. It was sonicated followed by filtration using Whatmann filter paper No. 1. The solutions were subject to analysis and results obtained as in **Table 4.**

Table 4 Analysis of Tablet formulation

Methods	Label Claim (mg)	%Amount found	%RSD (n = 6)
Normal	300	101.09	0.63
Reverse	300	100.28	1.25

Specificity

a) Normal phase

The specificity of the method was ascertained by analyzing standard drug and sample. The mobile phase resolved the drugs very efficiently. The R_f value of TRI was found to be 0.40. The peak purity of TRI extracted from tablet and standard TRI was tested at the peak - start (S), peak - apex (A) and at the peak - end (E) position (Figure 3 a).

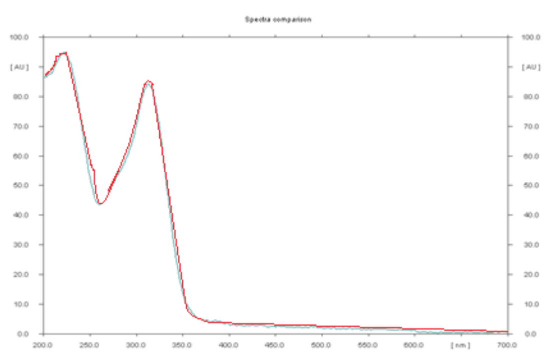

Figure 3(a) Peak purity spectra of standard TRI, sample 1 extracted from Triflusal capsules, scanned at the peak-start, peak- apex and peak-end positions of the band (Correlation > 0.99)

b) Reverse phase

The mobile phase designed for the method resolved the drugs very efficiently. The R_f value was found to be 0.58. The peak purity of TRI extracted from capsule and standard TRI was tested at the peak - start (S), peak - apex (A) and at peak - end (E) positions (Figure 3 b).

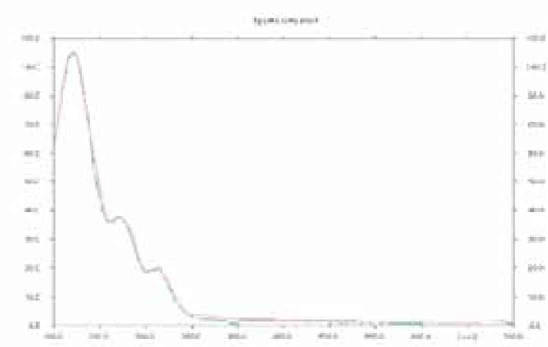

Figure 3 (b) Peak purity spectra of standard TRI, sample 2 extracted from Triflusal capsules, scanned at the peak-start, peak- apex and peak-end positions of the band (Correlation > 0.99)

Ruggedness and Robustness

Ruggedness of the both normal and reverse phase was performed for TRI by two different analysts maintaining similar experimental and environmental conditions.

Robustness was performed by introducing various small deliberate changes in the existing chromatographic conditions and effects on the results were examined for both methods.

Sensitivity

The sensitivity of measurements of TRI by the use of the proposed method was estimated in terms of the Limit of Quantitation (LOQ) and the lowest concentration detected under the chromatographic conditions as the Limit of Detection (LOD).

LOQ and LOD were calculated by the use of equation LOD = 3.3 x N/B and LOQ = 10 x N/B, where 'N' is standard deviation of the peak areas of the drugs (n=3), taken as a measure of noise, and 'B' is the slope of the corresponding calibration curve. The results were recorded for both the methods. Different validation parameters for the both methods for determining TRI content was summarized in **Table 5.**

Table 5 LOD and LOQ of the method

Drug	Normal phase		Reverse phase	
	LOD	LOQ	LOD	LOQ
TRI	98.55	295.65	1006.32	3018.96

NP-HPTLC versus RP- HPTLC

Two test differences between the proposed NP- HPTLC and RP- HPTLC method statistical tests were performed for the level of confidence 95% (**P** = 0.05) . Two way ANOVA was applied to test both method–sample interactions (interaction variation) and differences in the method precision since the within cell variation (residual variation) is greater than interaction variation as well as plate variations, the method–sample interaction and the differences between the methods are not significant.

Results and Discussion

An NP- HPTLC and RP-HPTLC methods was optimized with a view to develop an accurate and reproducible method so as to resolve drugs. Optimization of method was done by altering almost all the chromatographic conditions and the effect on R_f and peak shape were monitored for the drugs selected i.e. TRI. The samples were applied on the plates as bands, under continuous flow of nitrogen, by means of a CAMAG (Muttenz, Switzerland) Linomat-5 sample applicator fitted with a 100-µL syringe. Finally, for normal phase, **Toluene: Methanol: Triethylamine (8: 2: 0.5 v/v/v)** and reverse phase **methanol: water: triethylamine (6:4:0.5 v/v)** showed well- defined peak when the chamber was saturated with mobile phase for 15 min at room temperature. There is no tailing observed when plate was scanned at 314 nm. And for reverse phase Methanol: water: triethylamine in the ratio (6:4:0.5 **v/v**). The chamber saturation time was 30 min. The R_f for TRI were found to be 0.40 ± 0.02 and 0.58 ± 0.02, for both normal and reverse phase respectively. Mean recoveries of TRI for normal and reverse phase were 100.63 ± 15.03 and 100.12 ± 85.99 respectively.% RSD was less than 2 in intraday, interday precision and all parameters of robustness are in the limit. So the proposed method is more precise, accurate and robust **Table 6.**

Table 6 Validation parameters

Method Parameters	Normal	Reverse
Linearity (correlation coefficient)	0.999	0.999
Slope	0.901	0.205
Intercept	341.0	581.9
Ruggedness [% RSD]		
Analyst I [n=6]	0.39	0.96
Analyst II [n=6]	0.21	0.61
Robustness [% RSD] [n=6]		
Mobile phase composition	0.63	1.25
Duration of saturation time	0.73	1.69
Mobile phase volume	0.85	1.76

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Development distance	0.68	1.58
Sensitivity		
Limit of Detection (ng)	9852	1006.32
Limit of Quantitation (ng)	295.65	3018.96
Precision [%RSD]		
Intra-day [n = 3]	0.58 - 0.70	1.21- 1.63
Inter-day [n = 3]	0.39 – 0.82	1.39- 1.86
Repeatability [n = 6]	0.60	1.09

NP-HPTLC versus RP- HPTLC

Two test means (averages) a paired *t*-test was applied. The test removes any variations between samples the obtained value of *t* stat is lower than two tail, which leads to the conclusion that there is no significant difference between the means. The results of two way ANOVA and paired *t*-test are given in **(Table 8a, 8b and 8c)** respectively.

Table 8 (a) Two-way ANOVA test of TRI determination in six independent samples by NP-HPTLC and RP- HPTLC

Sample	NP- HPTLC	RP- HPTLC
1	99.72	99.50
2	100.27	101.41
3	101.16	101.60
4	101.77	101.92
5	100.83	100.42
6	101.16	101.63
Mean	100.82	100.91

Table 8 (b) ANOVA: Two factors with replications

Particulars	Normal (TRI)	Reverse (TRI)
Count	6	6
Sum	604.93	604.49
Average	100.82	100.91
% of total variation	4.35	11.89
P – value	0.26	0.22
T	0.7391	1.272
Stand Deviation	0.69	0.98
Stand error	0.26	0.37
Significance	Not	Not
F value	1.52	1.292

Table 8 (c) Average results of Triflusal determination by NP- HPTLC and RP- HPTLC and their correlation by paired *t*-test

Particulars	TRI
Count	12
Sum	1210
Average	100.83
% of total variation	0.42
P – value	0.47
T	0.2258
Stand Deviation	0.86
Stand error	0.32
Significance	Not significant
F value	1.695

Conclusion

The modalities adopted in experiment were successfully validated as per ICH guidelines. The proposed normal and reverse phase was validated by preliminary analysis of standard sample and by recovery studies for the determination of TRI in bulk and in capsule dosage form. For normal and reverse phase the percentage of average recoveries for TRI was obtained 100.63 and 100.12 respectively.

The proposed normal and reverse phase provide simple, rapid, accurate, precise and specific. It was observed that all the values are within the limits. The statistical evaluation of the proposed method was revealed its good linearity, reproducibility and its validation for different parameters and let us to the conclusion that it could be used for the rapid and reliable determination of TRI in capsule formulation.

Six real samples of capsules were determined simultaneously by NP - HPTLC and RP - HPTLC methods and the results were correlated. Statistical tests indicate that the proposed NP - HPTLC and RP - HPTLC methods reduce the duration of analysis and appear to be equally suitable for routine determination of Triflusal in pharmaceutical formulation. Thus the proposed method can be used for routine analysis of triflusal alone and also in combination; likewise the same can be applied to other formulations. Future plan includes further evaluation of degradants & stability indicating method.

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