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HPTLC METHOD FOR DETERMINATION OF TORSEMIDE IN HUMAN PLASMA

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ABSTRACT
A simple, selective and sensitive high performance thin layer chromatographic method for the determination of torsemide in human plasma is developed and validated. After precipitation of plasma proteins with methanol, the protein-free supernatant was spotted on plates precoated with silica gel 60 F254. The mobile phase consisted of a mixture of Ethylacetate: chloroform: Methanol in the ratio of 4:2:4 v/v/v. The drug showed considerable absorbance at 286 nm. The method was found to be linear over the concentration range of 1-7 µg/mL. Mean drug recovery was found to be 99.05%. Torsemide in plasma samples was stable when stored for different stability conditions. The method was found to be precise and accurate.

KEYWORDS: Torsemide, HPTLC, Human Plasma, Bioanalytical Method Validation.

INTRODUCTION
Torsemide[1,2] chemically 3-[4-[(3-methyl phenyl) amino] pyridine – 3-yl] sulfonyl -1- propan -2- yl urea (Fig.1), is a loop diuretic mainly used in the management of edema associated with congestive heart failure. It is also used at low doses for the management of hypertension.[3,4] Torsemide acts by inhibiting the $Na^+/K^+/2Cl^-$ carrier system[5] in the lumen of the thick ascending portion of the loop of Henle, resulting in the decrease in reabsorption of sodium and chloride.[6-7] In literature few analytical methods such as RP-HPLC method[8,9,10], UV spectropotometric method[11] have been reported for the determination of Torsemide. To the best of our knowledge, no High Performance Thin Layer Chromatographic (HPTLC) method has been reported for determination of Torsemide in human plasma. The present method describes a simple, selective and sensitive HPTLC method with a calibration range of 1-7 µg/mL for Torsemide in human plasma. The method utilizes protein precipitation with methanol as the sample preparation technique. The mobile phase employed was Ethylacetate:Chloroform: Methanol (4:2:4 v/v/v). The method was validated as per MHLW Japanese Guidelines.[12]

Chemical structure of Torsemide

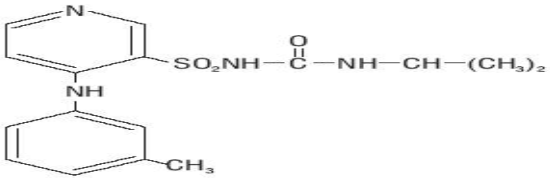

Figure. 1: Chemical Structure of Torsemide.

MATERIALS AND METHODS
Reagents and Chemicals
Working standard of Torsemide were obtained from Alkem laboratories Mumbai (India). Methanol AR Grade, Chloroform AR Grade, Ethylacetate AR Grade, were purchased from Merck specialties Pvt. Ltd. (Mumbai, India). Pooled plasma procured from local blood bank

Instrumentation and chromatographic conditions
Precise analytical weighing balance (Shimadzu AY120) was used for weighing. Chromatographic separation of drug was performed using aluminium plate precoated with silica gel 60 F_{254} (10 ×10) with 250 µm thickness (E. MERCK, Darmstadt, Germany) using a CAMAG Linomat 5 sample applicator (Switzerland). Samples were applied on the plate as a band with 5 mm width using CAMAG 100 µL sample syringe (Hamilton, Switzerland). Linear ascending development was carried out in 10 x 10 cm twin trough glass chamber (CAMAG, Muttenz, Switzerland) by using as mobile Ethylacetate: chloroform: Methanol in the ratio of 4:2:4 v/v/v. The optimized chamber saturation time for mobile phase was 15 min. The length of chromatogram run was 9 cm and development time was approximately 15 min. TLC plates were dried in a current of air. Densitometric scanning was performed on CAMAG thin layer chromatography scanner at 286 nm operated by WINCATS software version 1.4.2. The source of radiation utilized was deuterium lamp emitting a continuous UV spectrum between 200 to 400 nm.

Method Development

Selection of Mobile Phase

Different mobile phase like Methanol, Ethylacetate and Chloroform were tried in different ratio in order to find optimum conditions for Torsemide. After several trials Ethylacetate: chloroform: Methanol (4:2:4 v/v/v) was chosen as mobile phase for analysis in which optimum system suitability parameters were obtained.

Preparation of stock solutions of Torsemide

Stock solution was prepared by dissolving 25mg Torsemide in Methanol and then diluted to the mark with Methanol in 25 mL volumetric flask to get concentration of 1000 µg/mL. Working stock solution for Torsemide was prepared by diluting appropriately stock solution for Torsmide to get final concentration 100µg/mL.

Preparation of Spiked plasma sample

The reported peak plasma concentration values for Torsemide is 3µg/ml. On this basis, the linearity range was chosen as 1-7 µg/mL. Spiked plasma was prepared by spiking 4.8 mL plasma with 0.2mL of stock solution (25, 50, 75, 100, 125, 150,175 µg/mL). The content were mixed by Vortex mixer for 5 min. 1 mL of this solution was pipetted into separate test tube to which 1mL Methanol was added. These solutions were thoroughly mixed by Vortex Mixer and then were centrifuged for 15 min at high speed. The clear supernatant was injected. A blank plasma sample was treated similarly. 40 µl of supernatant from each tube were applied in the form of band on TLC plate.

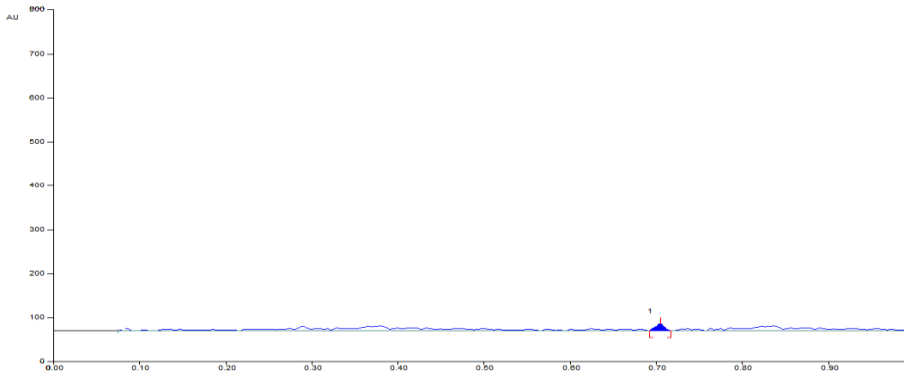

Fig. 2: Densitogram of blank human plasma.

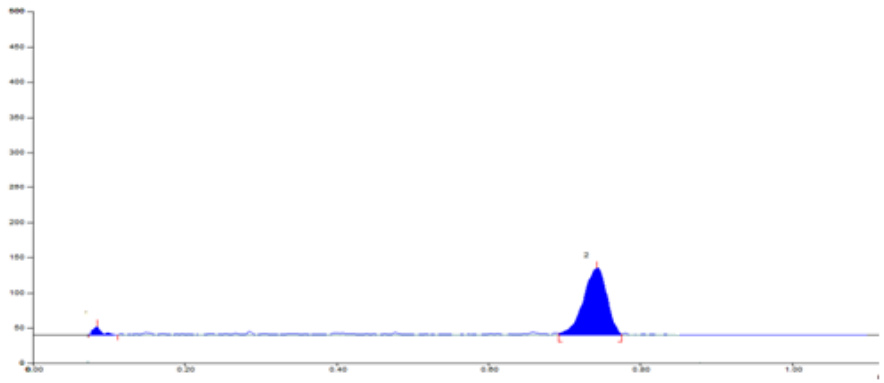

Fig. 3: Densitogram of Torsemide Rf 0.73 (140ng/band).

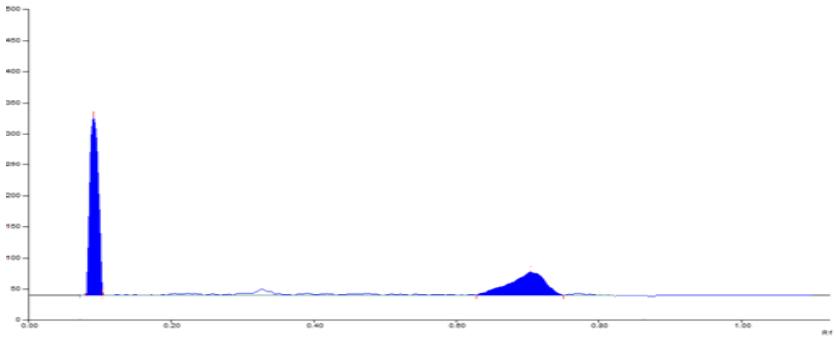

Fig. 4: Densitogram of human plasma spiked with Torsemide Rf 0.73 (140ng/band).

Selection of Wavelength
From standard stock solution further dilutions were done using methanol and it was scanned over the range 200-400 nm and spectra were taken. It was observed that drug show considerable absorbance at 286 nm.

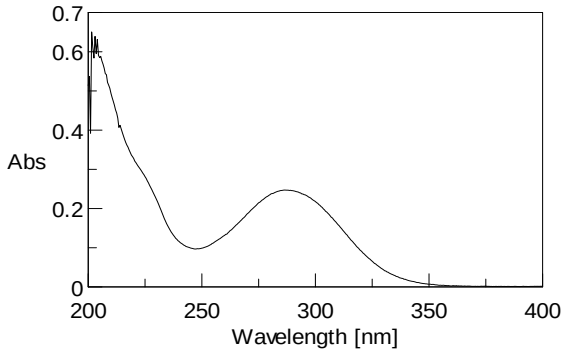

Fig. 5: UV Spectrum of Torsemide (10µg/mL).

Method Validation
The method was validated in accordance with MHLW guidelines, Japan. According to this guidelines there are parameters for bioanalytical method validation are.

1. Selectivity
The selectivity of the method was evaluated by analyzing pooled plasma samples spiked at LLOQ.

2. Calibration Curve
Linearity was tested for the range concentration 1-7µg/ml. Each sample in 6 replicates was analyzed and peak area were recorded.

3. Accuracy and Precision
Accuracy was measured by using minimum 5 determination per 4 concentration i.e. at LLOQ, LQC, MQC, HQC. The precision of this method was evaluated by % CV at different concentration levels.

4. Recovery
It was evaluated by replicate analysis of at least 3 times each at 3 concentration levels (low-, mid-, and high-levels).

5. Stability Study
The stability of the Torsemide solutions and plasma samples was also evaluated during method validation. Torsemide stability was evaluated using two concentration levels i.e. at LQC, HQC. 3 Types of stability studies were performed i.e. Freeze and Thaw stability, Short term stability and Long term stability.

Short term stability
A stock solution was kept at room temperature for 4 hours and checked for its stability.

Long term stability
A stock solution was kept in deep freezer for 30 days and checked for its stability.

Freeze thaw stability
The stability of low and high quality concentration samples was determined after three freeze thaw cycles. The percent degradation was determined by comparing area.

RESULTS AND DISCUSSION
The method was validated in terms of limit of quantification, Recovery, Selectivity, Precision, accuracy and stability.

1. Selectivity
It was evaluated using blank plasma samples. The absence of interference at Torsemide retention time was confirmed as shown in Table 1.

Table. 1: Results for selectivity.

Replicate No.	Nominal concentration (LLOQ) (20 ng/band)		
	Area of Torsemide	Calculated Concentration	
		ng/band	% Recovery
1	727.5	19.76	98.83
2	727.5	19.76	98.83
3	739.5	20.75	103.78
4	736.23	20.48	102.43
5	732.33	20.16	100.82
6	733.22	20.23	101.19
Mean		20.182	100.98
SD		0.392	1.96
%CV		1.94	

2. Linearity: Linearity was validated over a range of 1-7µg/ml. Best fit line equation was y = 12.111x + 488.1, the correlation coefficient for Plasma spiked with Torsemide (r2) 0.9973. Representative calibration curve is shown in Figure.5, as shown in Table 2.

Table. 2: Linearity Studies of Torsemide.

Sr. No.	Conc. (ng/band)	Area	S.D. (For n=6)	% Recovery
1	20	743.9	12.82	105.63
2	40	1002.98	9.531	105.81
3	60	1187.8	47.92	96.28
4	80	1422.75	75.17	95.07
5	100	1702.68	87.89	100.2
6	120	1921.82	63.70	98.65
7	140	2218.6	87.98	102.08
R^2	0.997			

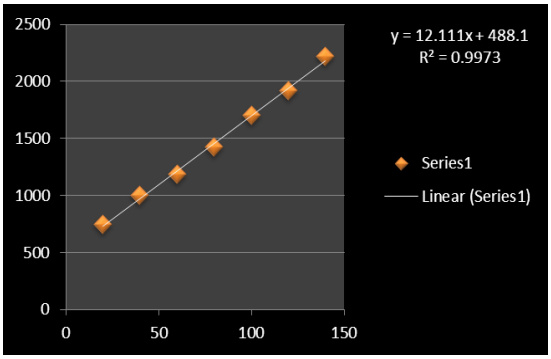

Fig.6: Calibration curve of Torsemide

3. Accuracy

Accuracy was measured by using minimum 5 determination per 3 concentration i.e. at LQC, MQC, HQC level ranged from 99.77%-96.91%, which is with in acceptance limit 85%-115% while at LLOQ level ranged from 99.04 which is with in acceptance limit 80%-120%.

Table. 3: Results of Accuracy for Torsemide.

Replicate No.	Nominal concentration			
	LLOQ	LQC	MQC	HQC
	20	60	80	100
	ng/band			
1	18.91	57.92	81.93	99.05
2	20.53	59.09	74.68	97.72
3	19.36	58.65	77.94	97.62
4	18.53	57.31	78.56	101.76
5	21.45	57.98	79.46	99.02
Mean	19.77	58.24	78.51	99.04
SD	1.215	0.784	2.626	1.93
%CV	6.14	1.36	3.34	1.94
% mean accuracy	99.04	99.77	96.91	97.37

4. Precision

A) Inter day Precision

The Inter day Precision was evaluated in five replicates for four different concentration of Torsemide on three consecutive days (fresh samples were prepared every day.) The % CV of calculated concentration for all quality control samples of LLOQ, LQC, MQC, HQC concentration level ranged from 1.55-14.89.

Table. 4: Results of Precision (Interday) for Torsemide.

Conc. Level	% CV		
	DAY 1	DAY 2	DAY 3
At LLOQ	4.13	14.89	5.59
At LQC	4.66	6.59	4.66
At MQC	2.30	1.74	1.81
At HQC	3.82	164	1.55

B) Intraday precision: Repeatability of the method was evaluated in five replicates on the same day for four different concentration of Torsemide (20,60,80,100 ng/band). The % CV of calculated concentrations for all quality control samples at LQC, MQC, HQC concentration levels ranged from, which is within acceptance limit 15%, and at LLOQ levels ranged from 1.34-6.14, summarized in table 5.

Table. 5: Results of Precision (Intraday) for Torsemide.

Conc. Level	At LLOQ	At LQC	At MQC	At HQC
% CV	6.14	1.36	3.34	1.94

5. Recovery

Recovery was evaluated by replicate analysis of at least 3 times each at 3 concentration levels (low-, mid-, and high-levels).

Table. 6: Results of Recovery for Torsemide.

Conc. Level	Area		% Recovery
Standard	Standard	Spiked Plasma	
LQC	1212.633	1194.167	98.47
MQC	1445.677	1434.233	99.20
HQC	1692.097	1683.09	99.46
Mean			99.05

6. Stability

Drug Stability in biological fluid is a function of storage conditions, chemical properties of drug, the matrix and the container system. Stability procedure should evaluate the stability of analyte during sample collection and handling after long term frozen at intended storage temp and short term (room temp.) storage conditions.

Freeze and thaw stability

Freeze and thaw stability of spiked quality control samples was determined after 3 Freeze and thaw cycles stored at -5 ^{0}C ±0 ^{0}C. Compared them again the freshly spiked quality control sample assessed stability. The mean % stability for HQC (100 ng/band) and LQC (60ng/band) was found to be 98.31% and 98.57% respectively, which is with in acceptance limit of 85-115%.

Long Term stability

Long Term stability of LQC and HQC was determined for period of 1 month after storing at 4 ^{0}C, comparing them against the freshly prepared stock solution assessed for stability. The % mean stability for HQC (100 ng/band) and LQC (60ng/band) was found to be 98.35%, 97.24% respectively, which is within the acceptance limit 85-115%.

Short term stability

Short term temp. stability of spiked quality control samples was determined for a period of 4 hrs. stored at room temperature. Comparing them against the freshly spiked quality control samples to assess stability. The % mean stability for HQC and LQC are found to be 98.45%, 97.24% respectively, which is within acceptance limit of 85-115%.

Table 7: Results of Stability for Torsemide

Stability	% Stability	
	At LQC	At HQC
Freeze and Thaw Stability	98.57	98.31
Long Term Stability	97.24	98.35
Short Term Stability	97.74	98.45

DISCUSSION

P. Thulasamma, have reported development and validation of RP-HPLC method for the quantitative estimation of Torsemide in pharmaceutical dosage forms and human serum. In this work plasma extraction technique used is protein precipitation using Acetonitrile whereas we have used methanol. The percent recovery from human serum sample Fairley matches that obtained in our work use of HPTLC in place of HPLC leads to high throughput advantages and rapid analysis.

CONCLUSION

In the study, rapid and sensitive HPTLC method has been developed for the determination of Torsemide in human plasma by protein precipitation technique. Validation results proved that the developed method performs well with selectivity, precision, accuracy, stability and linearity for the concentration range of Torsemide to be found in human plasma. The validated method covers the wide range of linearity over 1-7 µg/mL and is therefore suitable for the determination of Torsemide in human plasma at different therapeutic dose levels. The present method involves minimal sample pretreatment, resulting in fast analysis. Also it utilizes protein precipitation as the sample preparation technique, which eliminates the drawbacks of less recovery due to liquid-liquid extraction or the use of solid phase extraction cartridges which is relatively costly. The mean recovery of Torsemide was found to be 99.05%. HPTLC technique, offers advantage of high throughput, the present method is economical, simple and fast. The proposed method can be used for therapeutic drug monitoring in order to optimize drug dosage on an individual basis.

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