International Journal of Medicine and Pharmaceutical Research

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RESEARCH ARTICLE

Bioanalytical Method for Quantification of Torsemide in Human Urine Using LC-MS/MS

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ABSTRACT

Anaccurate and simple method was developed using high performance liquid chromatography with electron spray ionization tandem mass spectrometry (HPLC-ESI-MS) to quantify the concentration of torsemide in human urine. Stable isotobically labelled compound torsemide D7 was used as an internal standard (ISTD). The chromatographic analysis was conducted on a Zorbax C18 XDB column (100x 4.6mm,i.d5µm) within 3min, using methanol with 5mM ammonium acetate (70:30%, v/v) was used as mobile phase at the flow rate of 0.700mL/min under an isocratic condition. The ionization was performed on electron spray ionization interference with positive mode by multiple reactions monitoring (MRM). The mass transitions were 349.100→264.100 m/z for torsemide and 356.200→264.200 m/z for ISTD. Method validated as per USFDA guidelines and calibration curve was found to be linear in the range of 10.443-5000.411ng/mL. The results were within the acceptance limits. The extraction efficiency was 91.46% at the three quality control levels. The lower limit of detection (LLOQ) was found to be 10.443ng/mL. Stability studies demonstrated that torsemide was stable in urine during bench-top (3hr25min at room temperature), auto-sampler (23hr 20 min at 4°C), freeze-thaw (5cycles) and long termanalyte stability in urine (48days at -20°C).

Keywords: Torsemide, Human Urine, Stability, Protein Precipitation, Validation.

ARTICLE INFO

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MS-ID: IJMPR4352

PAPER-QRCODE

ARTICLE HISTORY: Received 20 August 2019, **Accepted** 22 October 2019, **Available Online** 10 December 2019

Citation: S. Satheshkumar, *et al.* Bioanalytical Method for Quantification of Torsemide in Human Urine Using LC-MS/MS. *Int. J. Med. Pharm. Res., 2019, 7(6): 229-236.*

CONTENTS

1. Introduction

Torsemide belongs to the loop diuretic medication with highly albumin protein binding (>99%) and bioavailability 90% [1]. Chemically it is sulfonylurea class of N-[(isopropyl amino) carbonyl] -4-[(3-methylphenyl) amino] pyridine-3-sulfonamide (Fig:1a) and molecular formula $C_{16}H_{20}N_4O_3S$ with compound weight 348.42 g/mol [2]. The pharmacological act as loop diuretics mainly inhibit the Na+/ 2Cl- / K+ carrier from the luminal side of the cell. Compared with other loop diuretics, torasemide has a more prolonged diuretic effect than equipotent doses of furosemide and relatively decreased potassium loss. Mainly used for treatment of oedematous states such as congestive heart failure (CHF), liver cirrhosis, nephrotic syndrome and chronic renal failure. It also proved to be an effective antihypertensive drug even in low dosages [3-4].

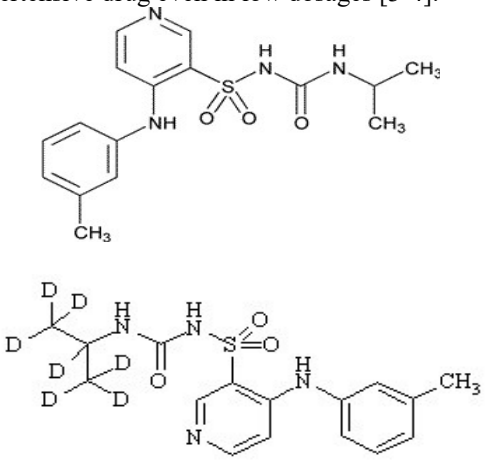


Fig. 1: Chemical structure of (a) Torsemide (b) Torsemide D7 (ISTD)

Drug literature review reveals that few analytical quantification methods have been reported for the torsemidein bulk, formulations, and biological matrices. Which includes UV spectrophotometric [5-6], high performance liquid chromatography [7-12] and ultra-high performance liquid chromatography tandem mass spectrometric detection (UPLC-MS/MS) [13-15]. The present work designed to develop a simple, rugged, economic and validated LC-MS/MS method for the determination of torsemide in human urine with deuterated internal standard torsemide D7(Fig:2a).

2. Materials and Methods

Chemicals and Reagents

The pure standard of torsemide (purity 99.68% by hplc) and torsemide D7 (Fig: 1b) (purity 99.24% by hplc) as is basis were purchased from Vivan life sciences hydrabad, India. Emparta grade of ammonium acetate, LC-MS grade of methanol, deionized milli-Q-weter and acetonitrile purchased from Merck Specialties Private Limited India, matrix: human urine.

Instrument and Equipment

Quantitative analysis was performed on an exion LC™ chromatographic system (AB Sciex, USA). The detection of analyte and ISTD performed using ESI and triple quadrupole mass spectrometer API 6500. Data acquisition and processing were performed by using analyst software version 1.6.3 (AB Sciex) to control all parameters of LC and mass spectrometry.

Chromatographic and MRM Condition

The chromatography separation of analyte was achieved by using zorbax eclipse XDB-C18 column (100×4.6 mm, 5µm) and the isocratic mobile phase consist of methanol: 5mM Ammonium acetate (70:30%, v/v) was delivered with flow rate of 0.700mL/min. The column compartment (oven) and autosampler temperature were at 30°C and 4°C, respectively with an injection volume of 5 µL. the analysis run time was completed within 5min.The main working parameters of the mass spectrometer are given in table 1.

Table 1: Multiple Reaction Monitoring (MRM) conditions

Parameters	Torsemide	Torsemide D7
General Dependent		
Mass spectrometer	API 6500	
Tuning mode	Manual	
Ion source	Turbo Ion Spray (ESI)	
Ionization Mode	Positive Ionization	
Spray needle set point (X/Y)	5/5	
Compound Dependent		
Transition (m/z) Q1→Q3	349.100→264.100	356.200 →264.200
Declustering Potential (V)	60	60
Entrance Potential (V)	10	10
Collision Energy (V)	30	30
Collision Cell Exit Potential (V)	15	15
Source Dependent		
Curtain Gas (psi)	40	
Ion Spray Voltage (V)	5500	
Temperature (°C)	500	
Gas Source 1 (psi)	40	
Gas Source 2 (psi)	45	
Collision gas (psi)	6	
Dwell Time Per Transition (msec)	200	

Sample preparation

The sample preparation was performed by protein precipitation method. Exactly 0.100 mL of urine sample was aliquoted and transferred into a 5mL tarsons RIA vial polypropylene tube and 0.050 mL of ISTD (1000ng/mL) working concentration solution was added, except for standard blank, to which 0.050 mL of 60% methanol solution (v/v) was added and the mixture was vortexed for 30 sec. To this 0.600mL of 100% acetonitrile was added and vortexed for 5min. centrifuged the all samples for 10 min at 5000 rpm in 4ºC. Following centrifugation, the supernatant solution was transferred into auto sampler glass

loading vials and injected 5 µL of the sample into the chromatographic system.

Method Validation

Method validation was done as per the criteria of industrial guidance for bioanalytical method validation of USFDA [16].

System Suitability

System suitability was evaluated by analyzing6 repeated injections from same vial of standard aqueous mixture equivalent to an about middle concentration of the calibration curve of torsemide and working concentration of ISTD during the start of the method validation and at the start of the respective day. The area ratio and retention time (Analyte and ISTD) of system suitability has within the tolerance limits of 5% CV.

Carryover Effect

Carryover effect was performed in order to remove the carryover from the previous injection to the next injection. Extracted blank, LLOQ and ULOQ samples were prepared from biological matrix of human urine as mentioned above extraction process. These samples were injected in the sequnce of mobile phase, extracted blank (without analyte and ISTD), extracted LLOQ, extracted ULOQ and above extracted blank urine samples during the start of the method validation. The area of interfering peaks at the RT of analyte has ≤ 20% of area of extracted LLOQ and at the RT of ISTD have ≤ 5% of area of extracted LLOQ.

Selectivity/ Specificity

The selectivity of the method was evaluated by analyzing six different lots of human urine matrix. From each lot, blank and LLOQ were processed using the above extraction method. For specificity, interference from analyte was established by processed minimum of six individual matrix lot with MQC concentration level without ISTD and interference from ISTD was established by processed minimum of six individual matrix lot with working concentration of ISTD without analyte.

Sensitivity

Assessed the sensitivity in the terms of percentage accuracy and precision which was denoted by %CV. It was evaluated with the lower limit of quantification (LLOQ QC) 10.835ng/mL of quality control sample along with all precision and accuracy bathch. The tolerance limit of percentage accuracy within ±20 and %CV ≤20.

Calibration curve

Calibration curve was constructed by plotting the ratio of peak area of torsemide and torsemide D7against the nominal concentration of calibrators. The calibration curve were fitted by weighting factor $1/X^2$least square linear regression equation method (y=mX+c) which are distributed throughout the calibration curve range from 10.443to 5000.411ng/mL of torsemide. The curve constructed by using balnk, zero and nine non-zero standards10.443, 20.885, 41.770, 360.087, 900.218, 1500.363, 2500.605, 3500.287 and 5000.411ng/mL. The tolerance limit of calibration curve was a correlation coefficient (R^2) of 0.98 or greeter, and each back-calculated standard concentration have ±15% deviation from the nominal value with the exception of LLOQ, which was set at ±20%.

Precision and Accuracy

Precision and accuracy batch was calculated by analysing four batches. For P&A studies five concentration level of quality control samples were prepared as lower limit of quantification (LLOQ), lower quality qontrol (LQC), medium quality control (MQC), high quality control (HQC) and dilution integrated quality control (DIQC)equivalent to 10.835, 27.089, 1504.920, 3781.205 and 18906.026 ng/mL respectively, with six replicates each. The intra-run and inter-run precision (% CV) for LOQ, MQC, HQC and DIQC should be ≤ 15% except for LLOQ, which was set at ≤20% and the intra-run and inter-run accuracy for LQC, MQC, HQC and DIQC should be within ±15% except for LLOQ, which was set at within ±20%.

Recovery

The percentage extraction efficiency of torsemide from human urine was calculated by comparing the mean peak response of six extracted low, medium and high (27.089, 1504.920, and 3781.205ng/mL) respectively, quality control samples to the mean peak response of six post-extracted low, medium and high quality control samples with the same concentrations. The percentage extraction effiency of ISTD from human urine was calculated by comparing the mean peak area of the prepared extracted ISTD to the mean peak area of post extracted ISTD at the concentration level intended for use. The % recovery of analyte and ISTD has to be less than 110%.

Matrix Factor

Matrix factor was evaluated at LQC and HQC level by using six screened different lots of human urine matrix. To determine the matrix factor two sets of six blank matrices were processed using the above extraction method. Post extraction samples were prepared by the standard of LQC and HQC containing internal standard were spiked into the extracted black matrices. In the same way, standard aqueous solution equal to LQC and HQC concentration containing internal standard was prepared using diluent and mobile phase as injected single batch. The acceptance criteria for IS normalized matrix effect was that the %CV should be less than 15%.

Dilution Integrity

Dilution integrity was evaluted to ensure that samples could be diluted with screened blank matrix of human urine without affecting the final concentration. Torsemide spiked human urine samples were prepared at concentrations of 18906.026ng/mL, above the upper limit of the calibration range. These samples were further diluted with human pooled urine five times dilution in six replicates and analyzed with all P&A batch. The six replicates have a precision of ≤ 15%CV and accuracy of 100 ± 15%.

Ruggedness

The ruggedness of the method was assessed by the deliberate changes in the experimental state with a precision and accuracy batch. The batch was supervised using a similar chemistry type of column to another column manufacture (Phenomenex Luna C_{18}) and different analyst in the same laboratory.

Run Size Evaluation

Evaluate the run size during method validation, which should include the number of samples to be analyzed under

a run during actual study sample analysis. Establish run-size based on the chromatographic run time and analyte stability.

Reinjection Reproducibility

Reproducibility is the precision between two laboratories. It also represents the precision of the method under the same operating conditions over a short period. Re-injection reproducibility shall be evaluated by re-injecting anyone of the accepted P & A.

Stability experiments

The aim of determining the stability of torsemide in human urine performed viz. bench-top stability, freeze-thaw stability, auto-injector stability, wet extract stability, Long-term analyte stability in urine, stock and working solution stability studies were carried out by using six replicates of the lower and higher quality control samples. The stability was calculated by comparing the found concentration to the nominal concentration values against the freshly prepared calibration standard and bracketed run acceptance quality control (LQC, MQC and HQC) samples.

Stock and working solution stability

To assess the standard stock solution stability of analyte and ISTD, stability samples were prepared and maintained at 2-8^{o}C for 21 days. The percentage bias calculated mean peak area of of the stability standard stock solution of analyte and ISTD against the comparable freshly prepared standard stock solution of analyte and ISTD, then injected six replicates of fresh and stability samples at LLOQ and ULOQ level.

Bench-top stability

To determine the stability of analyte in human urine on the based-top condition, six replicates of stability quality control (LQC and HQC) samples were set separately at ambient temperature up to 3 hr 25min then extracted and qualified.

Freeze-Thaw stability

Freeze Thaw stability of analyte was evaluated by six replicates of stability quality control (LQC and HQC) samples were frozen at -20 degree in the deep freezer. The frozen urine samples containing the analyte thawed at room temperature for a minimum 1 hour followed by refrozen for minimum 12 hours. The stability quality control samples were exposed to 5FT cycles before being extracted and analyzed.

Auto-sampler stability

To determine the stability of processed sample in autosampler condition, six replicates of stability quality control (LQC and HQC) samples were processed and left in the autosampler rack up to 23 hours 20 minutes at 4^{o}C then injected and quantified.

Wet Extract stability

To determine the stability of wet extract, six replicates of stability quality control (LQC and HQC) samples were processed and stored at 2-8^{o}C refrigerator condition for 19 hours as wet extract form prior to loading into LC autosampler.

Long-term analyte stability in urine

To determine the long-term stability of analyte in urine, six replicates of 3 set stability quality control (LQC, HQC and DIQC) samples were stored at -20^{o}Cin the deep freezer for 48days after completion of stability duration extracted and analyzed. All stability experiments were stable if assay values were within the adequate tolerance of ±15% of accuracy and ≤15%CV of precision.

3. Results and Discussion

The ionization techniques of positive and negative MRM mode was tried using harvardsyringe pump was carried out to obtain Q1 and Q3 ion mass spectra of analyte and ISTD with electron spray ionization probe source and the signal intensity was good and higher in the positive mode of ionization tuning. For torsemide and torsemide D7, the highly sensitive transitions were detected from precursor ion m/z 349.100 to product ion (Fig:2 a, b) m/z 264.100 and precursor ion m/z 356.200to product ion m/z 264.200(Fig:3 a, b), respectively.

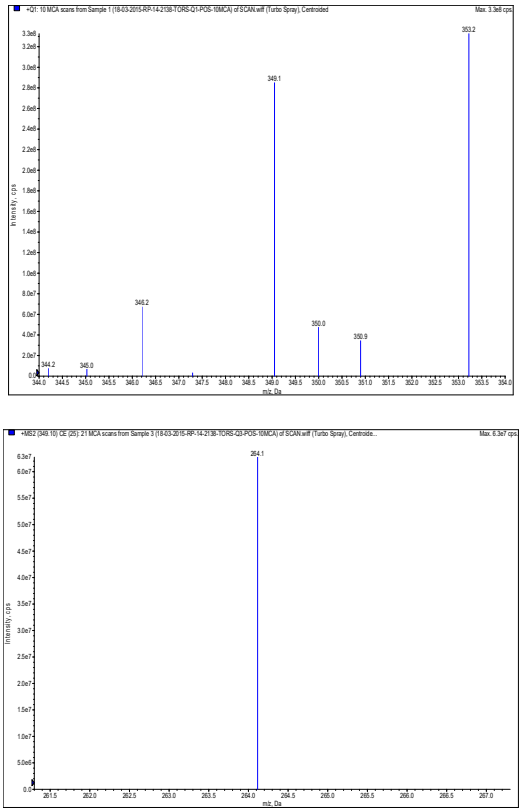
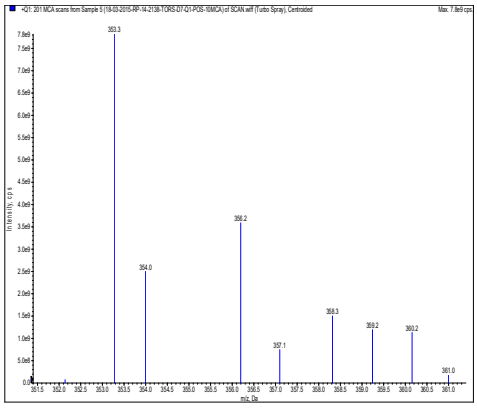


Fig. 2: Representative spectra for (a) Torsemide Q1, (b) Torsemide Q3



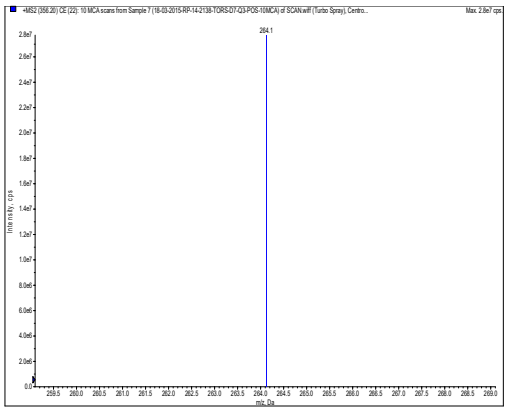


Fig. 3: Representative spectra for (a) Torsemide D7 Q1, (b) Torsemide D7 Q3

After many trails, the finest optimized conditions were attained with isocratic elution using reversed phase zorbax eclipse XDB-C18 column (100×4.6 mm, 5µm). a mixture of methanol: 5mM ammonium actate (70:30%, v/v) was used as the mobile phase operated at a flow rate of 0.700 mL per minute. The peak achieved were well defined symmetric peak shape and good response at lower concentration with the retention time of 2.37 min for analyte and 2.28 min for ISTD mode was suitable for the detection within a reasonable time of analysis less than 3 min.

Protein precipitation method was used for sample preparation since relativity inexpensive technique, good extraction efficiency as well as simple procedure. Methanol and acetonitrile precipitation solvents were tried, but hundred percent is acetonitrile was found to be most effective for extraction of both analyte and ISTD with minimal matrix effect and reproducible recovery. As a result of good response of selection in spiked LLOQ samples begins by the sample aliquote volume 100µL has been used. Thus, enhancing the sensitivity and accuracy of the LC-MS/MS analysis. These data indicate that the developed method is highly specific and selective for the analysis of torsemide in human urine samples.

System Suitability

The system suitability %CV of the retention time was found to be 0.57-1.81% for torsemide and 0.47-1.64% for torsemide D7. The %CV of the peak area ratio was found to be 2.04 to 3.77%. Prior to suitability few equilibration injections were given, and the results were found to be within the acceptance.

Carryover Effect

The results indicated that no carryover was observed throughout this chromatographic method for both torsemide and torsemide D7. It does not affect the precision and accuracy of the individual run.

Selectivity/ Specficity

Selectivity of the technique was verified on ten blank human urine samples obtained from different volunteers. The chromatographic method determined analyte of interest in the analyzed matrices without interference from endogenous components. This matrices lots were further selected for preparation of calibration curve and quality control samples. The % accuracy of individual lot's LLOQ samples were within the acceptable range of ±20%. The selectivity and specificity experiments ensured null interference at the retention time of analyte and ISTD. Results are given in table 2.

Linearity

The linearity of the method was demonstrated peak area ratio of analyte to ISTD was linear with reliable reproducibility over the concentration range of 10.443 to 5000.411ng/mL figure 4. At nine non-zero calibrator levels. The correlation coefficient R^2 for the calibration curve (Fig:5) ranged from 0.9997 - 0.9998 for torsemide. Results are given in table 3.

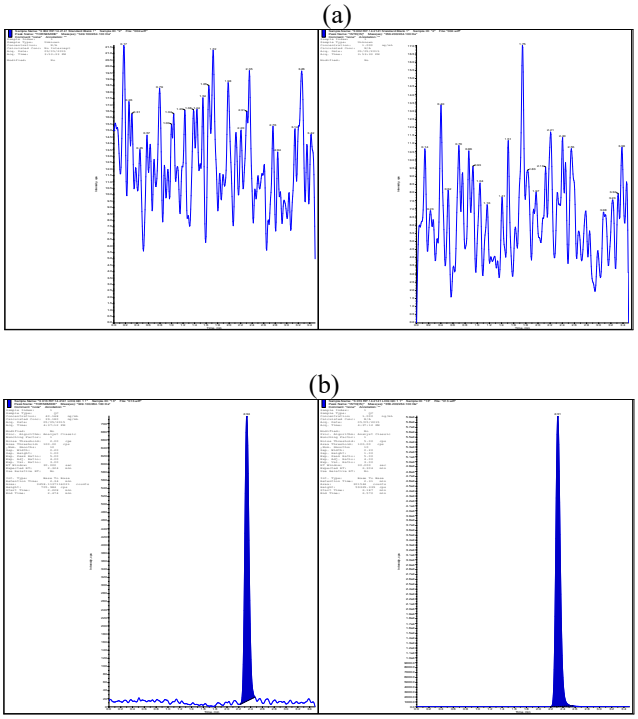


Fig. 4: Mass Chromatograms of (a) Blank urine, (b) Blank urine spiked with LLOQ

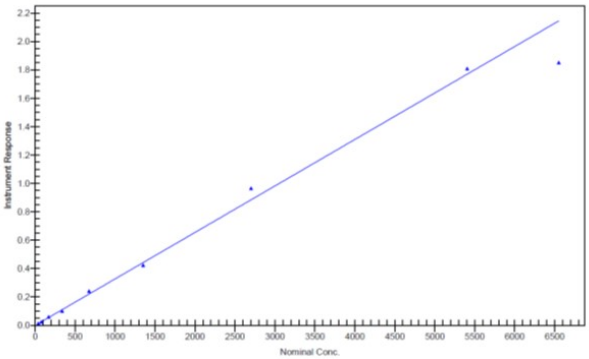


Fig. 5: Representative calibration curve for Torsemide in human Urine

Sensitivity/ Precision and Accuracy

The Precision and accuracy statistical data for QC's are summarized in table 4. The intra-run and inter-run precision for each concentration level within the range of 1.90 to 7.61%CV and 5.60 to 7.13 %CV, respectively and the intra

and inter run accuracy for each concentration level was within the range of 93.24% - 108.75% and 99.15% - 104.74% respectively. The lowest concentration with %CV less than 20% was taken as LLOQ and was found to be 10.443ng/mL. The result was summarized in table 4.

Recovery

The relative recovery for LQC, MQC and HQC of torsemide were found to be found 90.09%, 91.60% and 92.68% respectively. The percentage mean global recovery of analyte was found to be 91.46% with adequate precision of 1.42% CV and the ISTD percentage mean recovery was found to be 98.84%. The result data shows that the simple protein precipitation extraction procedure efficiently extracts torsemide as well as torsemideD7 from human urine. The results were summarized in table 5.

Matrix effect:

The post-extraction spiked method indicated that no significant effect of matrix ion was observed at the retention time of analyte and ISTD for QC levels (LQC and HQC). The %CV was found to be IS normalized matrix factor 2.00 and 3.03, correspondingly. The result of matrix effect as within the acceptable limit.

Dilution integrity: Dilution integrity of torsemide was performed up to five fold. The percentage nominal values was found within the acceptance limit of ±15% and the diluted samples mean precision was 1.90 to 5.91 % and accuracy was 95.97 to 108.75%.

Ruggedness

The present method was shown good ruggedness when it was performed by using different analyst and column of different manufacture. The accuracy and precision result was acceptable range of 93.97-101.34 % and 3.18-9.85 % CV respectively.

Stability: The stability of selexipag was assessed under different environment expected to be encountered during the analytical process and sample storage. The analyte passed all the stability parameter tests viz. stock solution stability (21 days at 2-8^{o}C), Auto-sampler (23h 20min at 4^{o}C), Bench-top (3h 25min), wet extract (19h at room temperature), Freeze-Thaw (5 cycles) and deep freezer stability (48 days at -20^{o}C). There was no significant decrease of the analyte concentration was observed. The summary of the stability parameters statistical data for torsemide presented in the table 6.

Table 2: Selectivity-Interference from endogenous compound for Analyte and ISTD

Sl. no	Biological Matrix ID	Area response					
		Analyte Area Blank)	Analyte Area (LLOQ)	%Inteference for Analyte	ISTD Area (Blank)	ISTD Area (LLOQ)	%Inteference for ISTD
1	Human Urine Lot-1	0	5984	0.00	0	469963	0.00
2	Human Urine Lot-2	0	6124	0.00	0	499353	0.00
3	Human Urine Lot-3	0	6325	0.00	0	473567	0.00
4	Human Urine Lot-4	0	6007	0.00	0	439766	0.00
5	Human Urine Lot-5	0	5418	0.00	0	436530	0.00
6	Human Urine Lot-6	0	5349	0.00	0	475243	0.00

Table 3: Linearity

Nominal Concentration (ng/mL)	STD-1 10.443	STD-2 20.885	STD-3 41.770	STD-4 360.087	STD-5 900.218	STD-6 1500.363	STD-7 2500.605	STD-8 3500.287	STD-9 5000.411
N*	4	4	4	4	4	4	4	4	4
Mean±SD	10.634	20.596	42.245	368.590	898.165	1521.353	2559.342	3616.073	5060.919
±SD	1.16	1.75	1.86	3.35	22.33	33.92	33.99	54.18	79.47
%CV	10.88	8.50	4.40	0.91	2.49	2.23	1.33	1.50	1.57
% Accuracy	101.83	98.62	101.14	102.36	99.77	101.40	102.35	103.31	101.21

*Number of each concentration injections

Table 4: Precision and Accuracy for Intra run and Inter run

P & A	LLOQ 0.100 ng/mL	LQC 0.285 ng/mL	MQC 15.845 ng/mL	HQC 34.446 ng/mL	DIQC 172.230 ng/mL
N*	**6**	**6**	**6**	**6**	**6**
Intra-run Mean	10.458	27.247	1538.266	3806.852	20436.572
Intra-run ±SD	0.66	2.07	97.50	240.67	876.73
Intra-run %CV	6.34	7.61	6.34	6.32	4.29
Intra-run % Accuracy	96.52	100.58	102.22	100.68	108.10
N*	**6**	**6**	**6**	**6**	**6**
Intra-run Mean	10.899	25.898	1605.719	3853.852	18143.974
Intra-run ±SD	1.01	0.98	91.78	271.21	1071.78
Intra-run %CV	9.27	3.78	5.72	7.04	5.91
Intra-run % Accuracy	100.59	95.60	106.70	101.92	95.97
N*	**6**	**6**	**6**	**6**	**6**
Intra-run Mean	10.827	27.559	1415.484	3836.715	20561.179
Intra-run ±SD	0.59	1.33	41.79	217.18	390.24
Intra-run %CV	5.46	4.83	2.95	5.66	1.90
Intra-run % Accuracy	99.92	101.74	94.06	101.47	108.75
N*	6	6	6	6	6
Intra-run Mean	10.226	26.726	1566.025	3525.599	20070.541
Intra-run ±SD	0.87	1.81	74.73	162.66	752.08
Intra-run %CV	8.54	6.79	4.77	4.61	3.75
Intra-run % Accuracy	94.38	98.66	104.06	93.24	106.16
N*	24	24	24	24	24
Inter-run Mean	10.603	26.857	1531.373	3755.754	19803.066
Inter-run ±SD	0.80	1.57	101.75	267.88	1108.68
Inter-run %CV	7.56	5.84	6.64	7.13	5.60
Inter-run % Accuracy	97.85	99.15	101.76	99.33	104.74

*Number of each concentration injections

Table 5: Recovery

QC ID	LQC		MQC		HQC		ISTD	
	Post Extracted Area	Extracted Area	Post Extracted Area	Extracted Area	Post Extracted area	Extracted Area	Post Extracted Area	Extracted Area
N*	6	6	6	6	6	6	6	6
Mean±SD	14040	12649	795506	728677	2189066	2028737	460221	436487
±SD	670	689	65938	27590	209956	126340	31821	28393
%CV	4.77	5.45	8.29	3.79	9.59	6.23	6.91	6.50
% Recovery	**90.09**		**91.60**		**92.68**		**94.84**	
%Global CV	**91.46**						**-**	
%Global recovery	**1.42**						**-**	

*Number of injections

Table 6: Stability

Stability Experiment	QC ID	Nominal concentration (ng/mL)	Concentration found (ng/mL) (mean ± SD)*	Precision (% CV)	Accuracy (%)
Bench top Stability	LQC	27.089	27.728	4.16	102.36
	HQC	3781.205	3658.552	1.34	96.76
Auto sampler Stability	LQC	27.089	26.407	5.90	97.48
	HQC	3781.205	3722.086	2.07	98.44
Wet extract Stability	LQC	27.089	27.089	3.05	100.00
	HQC	3781.205	3814.404	2.60	100.88
Freeze thaw Stability	LQC	27.089	27.033	5.59	99.79
	HQC	3781.205	3705.381	0.90	97.99

Reinjection Reproducibility	LQC	27.089	25.669	3.49	94.76
	HQC	3781.205	1592.644	4.17	105.83
Long term urine stability	LQC	27.089	25.437	3.69	93.90
	HQC	3781.205	3880.382	4.72	102.62
	DIQC	18906.026	18468.208	1.58	97.68

*Number of each concentration injections-6

4. Conclusion

A highly sensitive, selective, specific, accurate and precise LC-MS/MS method for the quantification of torsemide in human urine was developed. The extraction procedure of analyte in biological matrix simple with reproducible recovery and less matrix effect. Proposed chromatographic method was rapid, allowing for sample preparation procedure and analysis of a large number of sample in a short period of time and comprehensive method validation was carried out. All results were within the range of acceptable limits as specified in USFDA guidelines (2018). Hence, the developed method can be applied to PK and TDM studies in humans with desired precision and accuracy.

Authors Contributions

Both author have contributed equally.

Conflict of Interests

Declared none.

5. References

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