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Development and validation of stability indicating LC-MS/MS Technique for the quantification of tapentadol in biological matrices: Application to bioavailability study in healthy rabbits

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ABSTRACT

An Liquid chromatography tandem mass spectrometry (LC-MS/MS) technique is one of the best analytical methods for the quantification of drugs in biological samples. A stability-indicating analytical technique was developed for the quantitation of tapentadol in biological matrices as tapentadol with short runtime. Developed technique also suitable for bioavailability studies in healthy rabbits. Separation of tapentadol and tapentadol-d3 were achieved from plasma sample with solid-phase extraction and elution was processed with Luna-C_{18} (5 µ, 100 mm × 4.6 mm) stationary column with movable phase ratio comprising 2-mM ammonium acetate buffer (pH-3.6) and acetonitrile in the proportion of 10:90 % V/V. Quantitation was processed by processing the transitions of tapentadol and tapentadol-d3 at *m/z* 222.2 → 177.1 and 228.2 → 183.1, respectively, in positive ionization mode. Linearity was performed over the concentration range of 0.121 to 35.637 mg/ml ($R^2 > 0.99$) without matrix effect (2.74%). The inter- and intra-day precision findings were within 8.62% and 11.38%, respectively. Stability data showed that the tapentadol was stable when it exposed to different stability conditions. This technique was effectively applied to bioavailability studies of tapentadol in healthy rabbits.

INTRODUCTION

Tapentadol (TPD) is a synthetic analgesic drug which acts centrally. Analgesic activity of drug is due to µ-opioid agonist action and it prevents nor-epinephrine reuptake. Morphine is 18 times more potent than tapentadol to bind µ-opioid receptors and tapentadol is less effective in animals to induce analgesia. TPD increases noradrenaline concentrations by obstructing the nor-adrenaline reuptake at brain of the tats (Fidman and Nogid, 2010; Mahaparale and Samuel, 2015; Singh *et al*., 2013). TPD produce its analgesic effect without an active metabolite. It is chemically designated as 3-[(1R, 2*R*)-3-(dimethyl amino)-1- ethyl-2-methyl propyl] phenol hydrochloride.

An average absolute bioavailability is 32% approximately due to first-pass metabolism after single-dose administration. Maximum TPD serum concentration was observed after 1.25 hours (after dosing). A multiple dose (every 6 hours) study with vary in dose from 75 to 175 mg TPD showed an average of 1.6 accumulation factor to parent drug and 1.8 is for main metabolite (TPD-O-glucuronide), which were estimated primarily by medicating interval and half-life of TPD and drug metabolite (Leonhart, 2009; Raffa, 2012; Tzschentke *et al*., 2006; WHO, 2014).

In humans, TPD HCl metabolism is extensive, because 97% of parent drug is metabolized. Most of the drug is metabolized through Phase-2 path, and few amount metabolized through Phase-1 pathway (oxidative). The major metabolism pathway of TPD is glucuronic acid conjugation to yield glucuronide. After administration of drug by oral route, 70% (O-glucuronide—55.0%, sulfate of TPD—15%) of the drug dose is eliminated as conjugated form in the urine (Nossaman *et al*., 2010; WHO, 2014).

Literature review unveils that one Liquid chromatography tandem mass spectrometry (LC-MS/MS) (Coulter *et al*., 2010)

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method and two High performance liquid chromatography (HPLC) methods (Gaurang and Hitendra, 2013; Mahaparale and Samuel, 2015) were described for the quantification of TPD. No reported technique was on bioavailability study on healthy rabbits. The goal of the research was to develop a fast and sensitive bioanalytical technique for the quantitation of TPD in plasma samples and application of pharmacokinetics in healthy rabbits by LC-MS/MS.

MATERIALS AND METHODS

Chemicals and materials

TPD (LS, purity: 99.7, Batch No.: T3A03,) and TPD-d_3 (internal standard, Lot No.: CK-LT-365, purity: 98.85%) were bought from the MSN Labs, India, (chemical structure shown in Fig. 1) Acetonitrile and ammonium acetate were obtained from SD-Fine Chemicals, India. Acetic acid was acquired from MJ chemicals, Mumbai, India. Millipore water from Moscheim Cedex system utilized in processing of standard and sample solutions. SPE-cartridges were gained from Agela Technologies, China. The animal studies on healthy rabbits were approved by institutional ethical committee no-1292/ac/09/CPCSEA/17-43/A.

Liquid chromatography

Chromatographic separation achieved on LC-Shimadzu scientific instrument with Luna-C18 (5 µ, 100 mm × 4.6 mm) stationary column. Mobile phase ratio comprising ammonium acetate buffer (2 mM) and acetonitrile in the proportion of 10:90 % V/V was processed at 0.7 ml/minute flow rate. The chromatographic elution was completed in 4.5 minutes for each single analysis.

Mass system conditions

The mass detection was processed on mass system, MDS-Sciex atmospheric pressure chemical ionization (API)-4000 (Canada), furnished with electro-spray ionization ran in +MRM mode. The component and system source constraints were finalized through injecting TPD and TPD-d3 individual solutions into mass system. The optimized component parameters to monitoring TPD and TPD-d3 were as follows: Ccollision cell exit-potential: 6 V; entrance potential, 12 V; declustering potential, 36 V; and collision energy, 18 V. Mass instrument source conditions were optimized as: turbo-ion spray voltage, 5,000 V; nebulizer gas, 55 psi; collision-activated dissociation gas, 5 psi; heater gas, 45 psi; curtain gas, 18 psi and source temperature, 400 °C. Q1 and Q-3 were processed at unit resolution with dwell time of 200 ms for two analytes. Quantitation was processed by monitoring the transitions of tapentadol and tapentadol-d3 at *m*/*z* 222.2 → 177.1 and 228.2 → 183.1, respectively, in the positive ionization mode. Analyst software version 1.4.2 utilized for data acquisition and integration (MDS-Sciex, Canada) (Chen and Hsu, 2013; Jaivik *et al*., 2017; Patel *et al*., 2017).

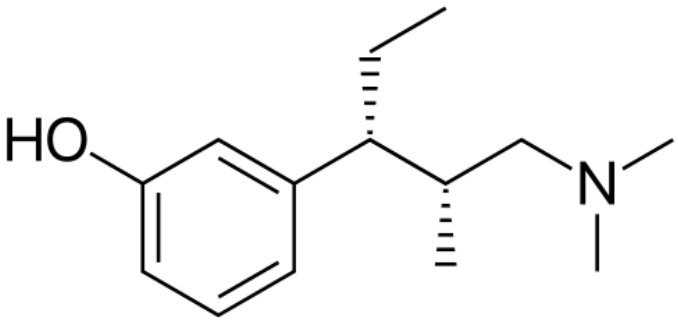

Figure 1. Structure of Tapentadol.

Protocol for stock, quality control, and calibration standard solutions

Two separate TPD-stock solutions were processed for CS (calibration standard solutions) and QC (quality control solutions) samples for the method validation and sample analysis. TPD and TPD-d3 stock solutions were processed with acetonitrile to get 1 mg/ml concentration. CS and QC working solutions were processed through suitable dilution with 50%V/V acetonitrile in water. Blank plasma (K_3EDTA) was infused into system before spiking to confirm that no matrix components interference at the retaining time of TPD and TPD-d3. An eight-point CS and QC-samples at four different concentrations were processed by spiking blank plasma with required quantity of TPD. CS solutions were prepared at concentration level of 0.1210, 0.3280, 1.6420, 4.1050, 10.2640, 17.1060, 28.5100, and 35.6370 ng/ml and quality control solutions at Lower limit of quantification quality control (LOQQC), Lower quality control (LQC), Medium quality control (MQC), and High quality control (HQC) at concentration level of 0.1220, 0.3590, 14.3580, and 28.7160 ng/ml, respectively (Badenhorst *et al*., 2000; Haritos and Ghabrial, 1999). The TPD-d3 working solution (150.0 ng/ml) was processed from TPD-d3-stock solution with 50%V/V acetonitrile in water.

Protocol for sample preparation

300 µl Plasma sample and 50 µl of TPD-d3 working solution (150 ng/ml) were transferred in ice-cold water bath and mixed well. To the resulting solution, buffer of 100 mM ammonium acetate (500 µl) was transferred and vortexed. The resulting samples were transferred into the pre-conditioned cartridge (Cleanert-PEP-3) and centrifuge (at 50,000 rpm) for 2 minutes at 2 to 10°C. The cartridges washed with 20% methanol (1 ml), and components were separated with 1 ml of acetonitrile. Then, extracted samples evaporated (at 40°C) to dryness under dry nitrogen stream utilizing Zymark Turbo-Vap-LV evaporator. Dried component was processed with 300 µl buffer of 5 mM ammonium acetate (pH-3.6) and acetonitrile in the proportion of 40:60 %V/V. 20 µl of resulting solution was infused into chromatographic system.

Method validation

The developed TPD-method was validated as per the guidelines of United States Food and Drug Administration (USFDA) and European Medicines Agency (EMEA) in human plasma. The technique was validated for specificity, precision, linearity, sensitivity, process efficiency, accuracy, re-injection reproducibility, matrix effect, dilution integrity, and stability study of TPD.

Selectivity and sensitivity

Selectivity of the method toward matrix metabolites, constituents, and associated medicaments were evaluated by screening 10 batches (2-lipemic, 2-haemolyzed, and 6-normal) of human plasma. The resulting processed samples were extracted with SPE and analyzed for TPD at limit of quantification (LOQ)

level. The peak response of all the matrix components in the blank sample at TPD and IS retention times should be <20 and 5% of average peak response of TPD and TPD-d3 in LOQ level, respectively. Sensitivity of the method estimated by assessing the ratio between signal and noise (S/N) in 10 batches of screened and spiked LOQ-samples (Fahimirad *et al*., 2016; Rana *et al*., 2008; Titier *et al*., 2008). The S/N was measured by the following formula:

$$\frac{S}{N}\text{ratio} = \frac{\text{Signal}:\text{noise ratio of LOQ}}{\text{average signal}:\text{noise ratio of blanks}} > 5$$

Precision and accuracy

The inter- and intra-day precision and accuracy were processed for TPD in human plasma. Within a day, intra-run and between days inter-run accuracy was analyzed six replica samples of LOQ-QC, low, medium, and high quality control levels. Method precision was evaluated by determination of % Relative standard deviation (RSD) for all quality control samples. The percentage deviation should be <15.0 (for LOQQC it should be <20.0). In the same way, the average accuracy should be ±15% (for LOQQC it should be ±20%) (Kirchherr and Kuhn, 2006).

Linearity

To prove the linearity of technique, three calibration curves were utilized. The peak area ratios of TPD were utilized to calculate regression coefficient. Least square ($1/X^2$) technique was utilized to determine the linearity curves individually. TPD concentrations were determined from each calibrator using back calculation technique. Regression coefficient finding should be $R^2 > 0.99$ desirable to all the linearity curves.

Process efficiency

TPD and TPD-d3 process efficiency (PE) at low, medium, and high quality control levels were estimated by observing the average peak area of TPD in six replica of extraction samples against the average peak area of TPD in un-extracted samples comprising TPD and TPD-d3 at concentration equals to those found in final TPD and TPD-d3 extraction samples. PE of TPD and TPD-d3 were measured by the following formula:

$$\%\,\text{PE} = \frac{\text{average peak area of analyte in extraction sample}}{\text{average peak area of analyte in un}-\text{extracted sample}} \times 100$$

Matrix effect

It was estimated in the form of absolute matrix effect (AME) measured by the following formula:

$$\%\,\text{AME} = \frac{\text{average peak area of analyte in post extraction sample}}{\text{average peak area of analyte in un}-\text{extracted sample}} \times 100$$

If AME value is one, it indicates that no interference of matrix components, less than one indicates ion suppression, and more than one indicates ion-enhancement (Titier *et al*., 2008).

Dilution integrity

It was processed by making the sample concentration nearly two times the 90% upper limit of quantification (ULOQ) concentration. The resultant solution was made dilution (two and four times) with blank plasma to get the solution concentration within the calibration range. Furthermore, the resulting samples were evaluated against fresh CS solution. The acceptance norms were same as precision and accuracy parameter.

Reinjection reproducibility

It was processed by reinjecting quality control samples from accepted precision and accuracy lot in the course of validation. The concentration of reinjected solutions was evaluated against the calibration standard solutions of the same precision accuracy lot, which were estimated 48 hours before. The percentage difference between re-injected and original values was measured by utilizing the formula:

$$\%\,\text{difference} = \frac{\left|\text{Original concentration} - \text{reinjected concentration}\right|}{\text{Original concentration}} \times 100$$

Stability

Low and High quality control samples (6) were regained from the freezer after three freeze and thaw cycles. Samples were stored at −30°C in three cycles of 24, 48, and 72 hours. For the long-term stability, QC samples were determined by analysis after 121 days of storage at −50°C. Bench-top stability was assessed for 6.5 hours period with standard concentrations. Stability solutions were prepared and extracted along with freshly spiked calibration standards. The accuracy and precision of the stability solutions should be ±15% of their nominal concentrations. The auto-sampler stability estimated after 72 hours under auto-sampler (at 10°C) condition. The freeze and thaw stability was performed by storing the QC samples at −50°C (frozen) and thawed at room conditions for three times. The change in analyte concentration was less (<15%) then the compound said to be stable (Kirchherr and Kuhn, 2006; Titier *et al*., 2008).

Pharmacokinetics in healthy rabbits

The kinetic constraints were measured by utilizing single dose by PK Solver from the results of plasma drug concentration verses time utilizing non-compartmental statistical method. The C_{max} and T_{max} were found directly from the concentration and time profiles. Linear trapezoidal technique was applied to estimate the AUC_0-t. The AUC 0-∞ was determined up to the last quantifiable concentration and the terminal elimination-rate constant (Ke). The Ke value was determined from the slope of the terminal exponential phase of the plasma of the linear regression method. The terminal elimination half-life ($t_{1/2}$) was calculated from 0.693/Ke.

RESULTS AND DISCUSSION

Internal standard selection

Identification and selection of IS was very important thing in an LC-MS/MS technique. The IS should have similar mass and chromatographic behavior with analyte to be determined. Stable isotope of particular analyte is the best and suitable IS for the technique. Therefore, TPD-d_3 isotope chosen for TPD internal standard.

Optimization mass conditions

Negative mode Electro-spray ionization (ESI) technique was less effective when compared with positive ionization mode. Primarily, mass constraints were tried in ESI and API sources, but better results were obtained in APCI source.

Optimization of chromatography

To get the better separation of TPD and TPD-d3, different kinds of phenyl and C_{18} stationary columns, such as Ascentis express, Hypurity-advance, Kinetex-ODS, Zorbax SB-ODS, Sunshell-C_{18}, Luna-ODS(2), ACE-ODS PFP, and kinetex-PFP were tried. TPD and IS were well separated using Luna-C_{18} 100A (5 µm, 100 × 4.6 mm) column. However, mobile phase ratio comprising buffer of ammonium acetate (2 mM) pH-3.6 and acetonitrile in the proportion of 10:90 % V/V was established optimal.

Method validation

Validation of the developed method was processed as per the regulatory guidelines and around was no nosiness detected at the retention time of TPD and TPD-d3 in the lots of plasma. The blank, blank with IS, LOQ, ULOQ and incurred sample chromatograms were represented in Figure 2. The S/N-ratio during the method validation was found to be more than 25, which was acceptable in accordance with the guidelines of EMEA and USFDA.

The drug has LOQ value of 0.1210 ng/ml and the precision and accuracy values were found to be 8.62% and 98.12% at LOQQC concentration level. The linearity graph was linear over the concentration levels of 0.1210–35.6370 ng/ml for TPD. Linearity curve was made using peak response ratio of drug to IS and the "R^2" value was estimated and the value was more than 0.99. Precision and accuracy were processed and the findings were tabulated in Table 1. The inter day and intra-day precision were measured in %RSD and the values were found between 1.730% and 11.380%, and the inter- and intra-day accuracy findings were present between 97.34% and 103.74%.

AME has an important role in ESI in mass system, which influences the ionization process of analyte by ion-suppression or

Table 1. Intra- and inter-day results.

QC solutions	% intra-day accuracy[1] (Mean ± SDng/ml)	% inter-day accuracy[2] (Mean ± SDng/ml)	%intra-day precision[3]	%inter-day precision
LOQQC (0.122 ng/ml)	97.34 (0.1188 ± 0.009)	98.12 (0.119 ± 0.0084)	11.38	8.62
LQC (0.359 ng/ml)	103.74 (0.372 ± 0.036)	102.24 (0.367 ± 0.04)	6.26	4.92
MQC (14.358 ng/ml)	102.88 (14.771 ± 1.005)	103.34 (14.837 ± 0.967)	2.29	1.91
HQC (28.716 ng/ml)	102.94 (29.56 ± 2.17)	103.50 (29.721 ± 1.892)	1.98	1.73

[1]n = 6.
[2]Values found from three runs (n = 18).
[3]n =6.

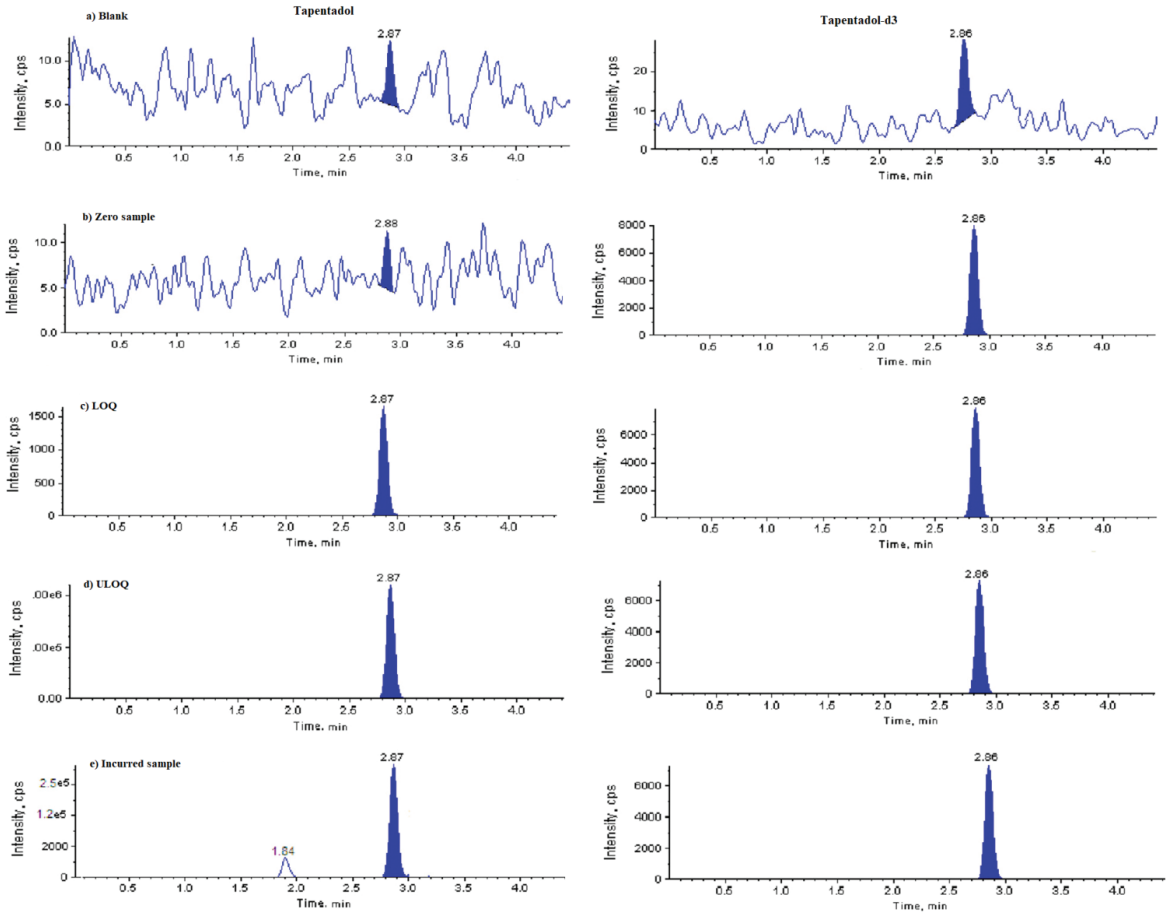

Figure 2. Representative chromatograms. (a) Blank, (b) TPD-d3, (c) Limit of quantification, (d) Upper LOQ, and (e) Incurred sample.

enhancement. The AME was evaluated at quality control level and the % RSD values were found in the range of 0.84 to 4.50. The findings shows that there was no effect of matrix components for analyte after the extraction of sample. The method PE for TPD and TPD-d3 were stable across all the quality control levels. An average process efficiency of TPD and IS were found to be 77.380% and 75.040%, respectively. The % RSD of average process efficiency over low, medium, and high quality controls was less than 3. The findings of AME and PE were represented in Table 2 and the relative matrix effect findings were tabulated in Table 3.

Stability studies of TPD were processed for Auto-sampler stability (10°C, 76.90 hours), Bench top stability (ice-cold water, 6.5 hours), Freeze and thaw stability (3 freeze and thaw cycle), Long term stability (−50°C, 121 days) and all the findings were shown in Table 4. The detected average nominal concentrations of TPD were within ±15% of their particular nominal concentration. There was no change in the concentration of TPD drug in human-K_3EDTA for 2.0 hours.

Method Reinjection reproducibility was proven by reinjecting quality control solutions of precision and accuracy lot-3 and quantified against the actual estimated linear graph of precision and accuracy lot-3. The % change for all the reinjected quality control solutions were ≤7.450.

Table 2. Tapentadol process efficiency and absolute matrix effect.

Quality control level	A[1] (%CV[2])	B[3] (%CV[2])	C[4] (% CV[2])	% AME[5]	% PE[6]
LQC	39,590 (3.6)	38,498 (4.3)	26,841 (4.1)	98.81	75.87
MQC	1,405,786 (1.6)	1,406,632 (4.3)	1,201,023(7.3)	99.35	79.91
HQC	2,812,201 (0.9)	2,701,598 (0.8)	2,109,915 (1.8)	97.26	76.54

[1]Average peak area of 6 replicates processed in re-constitution solution.
[2]Coefficient of variation.
[3]Average peak response of 6 replicates processed by spiking blank sample.
[4]Average peak area of 6 replicates processed by spiking pre-extraction.
[5]B/A × 100.
[6]C/A × 100.

Table 3. Tapentadol matrix effect.

Plasma batch	LOQQC (0.122 ng/ml)		HQC (28.716 ng/ml)	
	Average concentration (% CV[1])	% bias	Average concentration (% CV[1])	% bias
1	0.121 (1.2)	−0.82	28.799 (0.20)	0.29
2	0.122 (5.2)	0	28.632 (0.00)	−0.29
3	0.126 (4.5)	3.28	28.145 (1.80)	−1.99
4	0.118 (0.6)	−6.56	29.527 (0.70)	1.14
5	0.114 (4.4)	−3.28	29.042 (0.80)	2.82
6	0.121 (4.1)	−0.82	28.960 (0.40)	0.85

[1]Coefficient of variation.

Application of pharmacokinetic study

Developed and validated technique was utilized in the estimation of TPD in human plasma samples to study the kinetics of single oral dose of 300 µg/1.8 kg body weight (equivalent to 4 mg capsule) in six healthy rabbits. Graph was plotted by taking plasma concentration of TPD on *Y*-axis and time on *X*-axis, the results were represented in Figure 3. This plot was plotted by considering the data between 0 and 12 hours. TPD shown T_{max} of 2.27 ± 0.025 and mean $C_{max,}$ $AUC_{0®t}$ and $AUC_{0®a}$ for Test formulation is 294.82 ± 38.02, 852.72 ± 151.14, and 960.45 ± 205.069, respectively. The findings were shown in Tables 5 and 6.

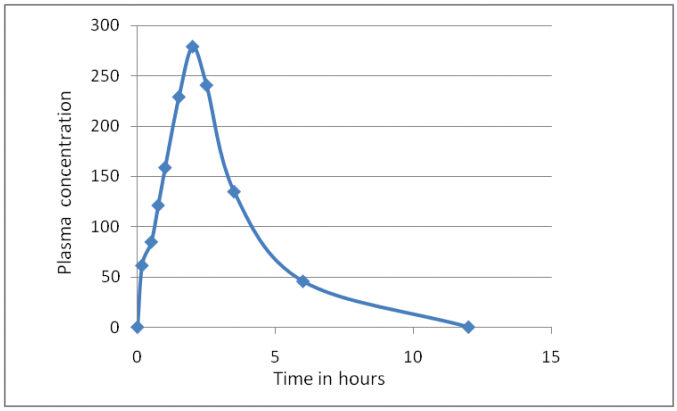

Figure 3. Plasma (mean) concentration of TPD—time profile curve.

Table 4. Tapentadol stability.

Stability type	Concentration level	Comparison sample (ng/ml)	% CV	Stability sample concentration	% CV	% Change
Bench top stability (ice-cold water for 6.5 hours)	LQC	0.358	1.46	0.359	2.6	2.64
	HQC	28.832	1.82	28.716	1.85	−0.57
Auto-sampler stability (10°C for 76.90 hours)	LQC	0.358	7	0.359	1.57	−1.95
	HQC	28.832	1.27	28.716	1.38	1.35
Long-term stability (−50°C for 121 days)	LQC	0.358	2.35	0.359	2.43	0
	HQC	28.832	0.63	28.716	0.96	3.94
Freeze/thaw stability	LQC	0.358	1.46	0.359	2.75	2.05
	HQC	28.832	1.82	28.716	1.85	0.36

CV: Coefficient of variation.

Table 5. Estimated plasma concentrations of healthy rabbits.

Estimated concentrations in ng/ml								
Time (hours)	**Rabbit-1**	**Rabbit-2**	**Rabbit-3**	**Rabbit-4**	**Rabbit-5**	**Rabbit-6**	**Mean**	**SD**
0	0	0	0	0	0	0	0	0
0.16	48	64	71	33	65	86	61.17	18.45
0.5	67	89	98	59	84	111	84.67	19.29
0.75	95	122	144	109	112	144	121.00	19.80
1	138	165	183	123	155	187	158.50	25.09
1.5	227	234	251	161	244	256	228.83	34.90
2	284	277	298	214	301	299	278.83	33.16
2.5	168	321	198	228	185	343	240.50	73.87
3.5	106	146	154	111	123	168	134.67	25.03
6	29	48	62	18	46	70	45.50	19.53
12	0	0	0	0	0	0	0	0

SD = Standard deviation.

Table 6. Test animals estimated average values of PK parameters.

Parameter	Unit	Mean value (n = 6)	SD
Lambda_z	1/h	0.48731	0.13558
$t_{1/2}$	H	1.50741	0.37908
T_{max}	H	2.27	0.27386
C_{max}	ng/ml	294.822	38.02
T_{lag}	H	0	0
C_{last_obs}/C_{max}		0.14926	0.05223
AUC 0-t	ng/ml*h	852.72	151.14
AUC 0-inf_obs	ng/ml*h	960.45	205.069
AUC 0-t/0-inf_obs		0.89669	0.04644
AUMC 0-inf_obs	ng/ml*h^2	3053.83	936.775
MRT 0-inf_obs	H	3.11926	0.36743
Vz/F_obs	(mg)/(ng/ml)	0.00296	0.00055
Cl/F_obs	(mg)/(ng/ml)/h	0.00141	0.00031

CONCLUSION

A rapid, specific, accurate, and sensitive LC-MS/MS technique was developed and validated to analyze tapentadol using tapentadol-d_3 as IS in human plasma. This validated LC-MS/MS technique can be useful in high throughput analysis and has been effectively applied to study the pharmacokinetics of tapentadol in rabbits. The intra-day precision and inter-day precision in % RSD ranged from 1.73% to 11.38% and the intra-day and inter-day accuracy ranged from 97.34% to 103.74%. The average process efficiency of TPD and TPD-d3 by this technique were 77.38% and 75.04 %, respectively. LOQ was 0.121 ng/ml of TPD in plasma. The precision and accuracy at LOQQC concentration were 8.620% and 98.120%, respectively. The pharmacokinetic studies of tapentadol have shown T_{max} of 2.27 ± 0.025 and mean $C_{max,}$ $AUC_{0–t}$ and $AUC_{0–a}$ for Test formulation is 294.82 ± 38.02, 852.72 ± 151.14, and 960.45 ± 205.069, respectively.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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