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ICH-Compliant Development and Validation of RP-HPLC and UV-Visible Spectrophotometric Methods for Quantitative Estimation of Tapentadol in Bulk and Biological Matrices

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Abstract

Background: Two novel methods, one an RP-HPLC technique and the other a chromogenic bioanalytical UV-Visible spectrophotometric method were developed and validated for the quantitative estimation of Tapentadol in bulk, pharmaceutical formulations, and human plasma. The bioanalytical method utilized a chromogenic reaction with Gibbs reagent to form a colored complex. Protein precipitation with methanol was used for the plasma extraction process for chromogenic bioanalytical method. Materials and methods: For Chromogenic Bioanalytical method plasma samples were prepared using protein precipitation with methanol. The reaction produced a blue complex measurable at 653 nm, while RP-HPLC detection was performed at 270 nm. The RP-HPLC method was validated over a concentration range of 15–105 µg/mL. The mobile phase consisted of 0.1% formic acid (pH 6.8) in water and methanol (80:20, v/v) with a flow rate of 0.7 mL/min at temperature of 25ºC. These methods were rigorously validated to ensure accuracy, precision, and reliability. Results: For HPLC the retention time was recorded at 2.478min, precision was below 2% and the accuracy for HPLC was 99.97%. LLOQ and ULQC samples of bioanalytical method was found to be 20 and 240 µg/mL. Precision, expressed as relative standard deviation (RSD), was consistently below 10%, with in acceptable limits and robustness of the method was within limits. Conclusion: Chromogenic bioanalytical method provides high sensitivity by using Gibbs reagent for quantifying Tapentadol in human plasma. Bioanalytical method offer reliable quantification of TPD in biological matrices. HPLC method demonstrates adequate validation for every parameter, including peak purity assessment as well, ruggedness as well robustness, the range, its specificity along with accuracy, linearity and precision in compliance with ICH criteria. The study's findings demonstrated that this method was suitable for routinely quality control determining tapentadol levels in prescribed dosage forms and bulk.

Keywords: Chromogenic Bioanalytical method, Tapentadol (TPD), RP-HPLC, UV-visible spectroscopy, Gibbs reagent.

Introduction

One type of centrally active analgesic that is recommended for moderate to severe pain is tapentadol. It reduces pain through both opioid and non-opioid receptors due to its special two ways of functioning, which involves blocking Reabsorbing norepinephrine thus performing as a substance known as agonist for mu-opioid receptors. 2,6-Dichloroquinone-4-chloroimide. It is mostly used for phenol measurement and identification. It reacts

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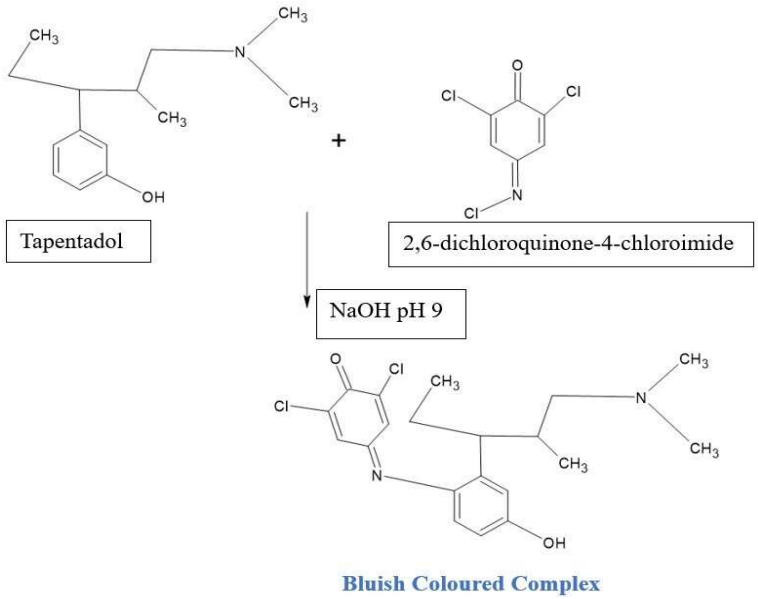

Fig. 1: Chemical Reaction of Tapentadol with Gibbs Reagent

severely to phenols. First, Dacre looked at the Gibb's reagent. The chemical molecule known as 2,6-Dichloroquinone-4-chloroimide, or the Gibbs reagent, is used as a colorimetric indicator for phenolic chemicals. (1-5) 2,6-dichlorophenolindophenol, a redox indicator, is created when phenol reacts with itself. Imines are created when Gibb's reagent is mixed with phenolic compounds. The 2, 6-dichloro quinone mono imines are produced by solvolyzing this reagent with Gibb's reagent. After attaching to the 2, 6-dichloro quinone mono imine, the para position of the phenolic compound joins with phenolic molecules to form the adduct as shown in (Fig. 1). Adduct deprotonates to produce a bright 2, 6-dichloro indophenols product with a wavelength of 500–670 nm. The entire procedure is unaffected by the medium's pH. It also interacts favorably to p-alkoxy phenols as well p-substituted aldehydes, including p-substituted halogen phenols. (6-8)

Materials and Methods

Tapentadol pure API was gift sample form the pharmaceutical company with 99.8% purity (w/w, anhydrous basis) Certificate of Analysis (CoA) was included with the standard. According to the manufacturer's instructions, this standard was used within the designated retest date and stored. Keeping all necessary records, such as the batch number, CoA, and procurement records, in accordance with GLP procedures guaranteed the traceability of the reference standard. Tapentadol tablet dosage form was purchased from a nearby pharmacy and for HPLC the HPLC-grade methanol in order formic acid along with distilled water utilized in this procedure. For chromogenic Bioanalytical UV–visible spectrophotometric method, 2,6-dichloroquinone-4-chloroimide or Gibbs reagent, Methanol, ethanol, 0.01M Sodium Hydroxide Solution, dilute HCL, Distilled water, human plasma samples were collected from a blood bank.

Handling of human plasma and organic solvents

Human plasma was considered infectious and was handled with universal caution. All waste was disposed in red bags as biohazardous waste. Organic solvents were gathered in appropriately labeled and

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segregated containers in accordance with hazardous waste management procedures. The disposal of solvents was never done down the drain; instead, EHS pickup procedures were used to handle trash.

Instrumentation and Chromatographic Conditions

RP-HPLC, Shimadzu, model UV-Visible Double-Beam Spectrophotometer 1800, and SYSTRONIC SS203. Digital weighing balances were used for sensitively weighing drugs. Spectra Treats for UV-visible spectrophotometer, LC solutions software, and a Shimadzu LC-20AD system were utilized for the HPLC analysis. A UV detector with a setting of 270 nm was used for the detection. For chromatographic separation, the Sunsil column of C18 ODS (150mm × 4.6mm as well 5 µm particle size) had been employed. Mobile phase has been made by adjusting water pH to 6.8 by formic acid that is in ratio of 0.1% Formic acid pH [6.8] in water: Methanol ratio of [80:20]. Mobile phase had been supplied with a 0.7 mL/min flow rate after being completely degassed before to use at ambient temperature of 25ºC.

Injector Precision and Justification of Injection Volume

A 20 µL injection volume was selected based on method optimization to ensure adequate sensitivity and peak shape without overloading the column. Injector repeatability was evaluated by performing six replicate injections of a mid-level standard solution (30 µg/mL). The %RSD of peak area was found to be less than 1.5%, demonstrating excellent injector precision and confirming the reliability of the chosen injection volume for routine analysis.

Development of the RP-HPLC methodology for tapentadol determination

Standard solution preparation: Pure API Tapentadol (0.01g) was properly weighed before and placed into a volumetric container with a capacity of 10 mL. 1000 µg/mL of stock was made by adding HPLC-grade methanol and make up the volume and then 1 mL from 1000 µg/mL was then dilute to the proper volume using methanol to create a 100 µg/mL working standard solution. The 15, 30, 45, 60, 75, 90 as well 105 µg/mL of different concentrations were obtained by serially diluting the working standard.

Selection of wavelength

An analysis of a sitagliptin mixture containing 10 µg/mL Tapentadol was carried out using a UV-visible spectrometer in scan mode. The wavelength was estimated by obtaining measurements between 200 and 400 nm. UV absorbance peak was observed at 270 nm.

Optimized method for analysis of Tapentadol

Chromatographic conditions were adjusted for the RP-HPLC procedure to efficiently separate and retain TPD. C18 ODS column (Sunsil, 150 mm x 4.6 mm, 5 µm) had been employed for analysis. HPLC-grade methanol along with (pH 6.8) Water with 0.1% formic acid had combined to create mobile phase entire process was executed in isocratic mode for a total of 10 minutes, employing a Sample or injection volume about 20µL as well as an injection flow rate about 0.7 mL/min. Accurate retention as well peak symmetry were indicated by the chromatogram's Gaussian peak. The efficiency of the column and the dependability of the procedure were confirmed by the retention time, The theoretical plate count was found to be above 2000, through a retention time period of 2.478 minutes.

HPLC validation parameters in accordance with Q2R2

Selectivity

Ensures no interfering peaks appear at the retention time of the analyte or internal standard. Blank mobile phase was injected.

Specificity

The specificity can be determined by looking at the interference caused by the improved method. Interference peaks shouldn't show up within blank samples when

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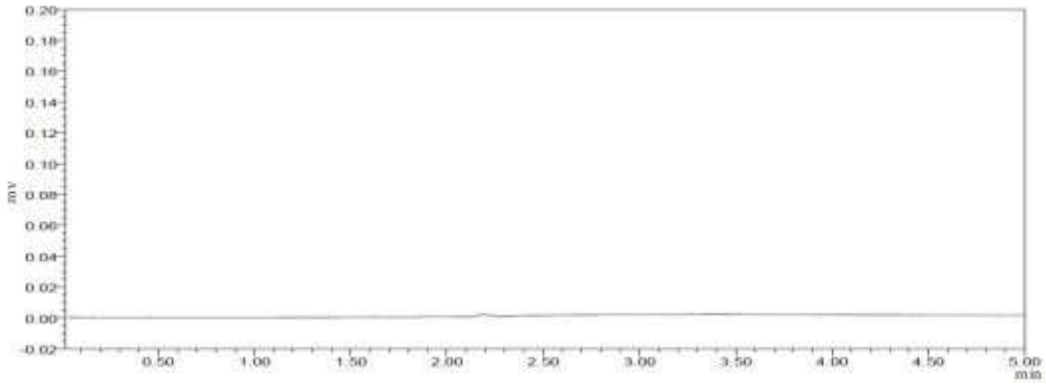

Fig. 2: Blank chromatogram of HPLC

using these medications. when employing this approach (9-10). Consequently, it was claimed that this method was distinct. By infusing blank solutions, specificity was achieved as shown in (Fig. 2). Tapentadol standard solution of 20 µg/mL was tested to confirm specificity.

Carry over

System carry-over, blank injections (mobile phase) were performed immediately after the injection of the highest concentration standard. These blank runs showed no detectable peaks at the retention time of Tapentadol, indicating the absence of residual analyte in the system. This confirms that the method is free from carry-over and suitable for sequential sample analysis without contamination risk.

Linearity

10 milligrams of pure API TPD was carefully weighed and dissolved in 10mL of HPLC-grade methanol in a freshly cleaned volumetric flask to produce 1,000 µg/mL of stock concentration. This stock solution 1mL was diluted utilizing methanol to provide the working standard to the proper level in another 10mL volumes flask, resulting in its optimum level of 100 µg/mL. Utilizing working standard quantities 15, 30, 45, 60, 75, 90 as well 105 µg/mL a series of dilutions had been made as indicated in (Figs. 3, 4 and Table 1).

Precision

0.6 mL of a 1000 µg/mL stock containing standard tapentadol had been delivered to 10 milliliter volume container, and volume was brought on with the HPLC-grade methanol to form a standard TPD of 60 µg/mL. To evaluate the accuracy of the procedure, this solution was made five times under identical circumstances. Inter-day accuracy was also evaluated. (11-15).

Accuracy

Accuracy of developed HPLC approach had been assessed through conducting recovery trials in triplicate (n=3) at three different levels of concentration for the calibration range. Known quantities of Tapentadol (TPD) were spiked into standard solutions at concentrations of 15, 30, and 45 µg/mL. For each level, 1mL of respective standard solution was combined with 1 mL of a 60 µg/mL sample solution. Obtained mixtures has been diluted to reach the final 10mL using the appropriate diluent. The recovery percentage for TPD at each concentration level was calculated, and the mean recovery values were determined. The accompanying Table 2 shows that every analysis had been carried out in triplicate.

LOD and LOQ

The term "detection limit" specifies the lowest possible level of analyte which can be effectively detected. Conversely, the

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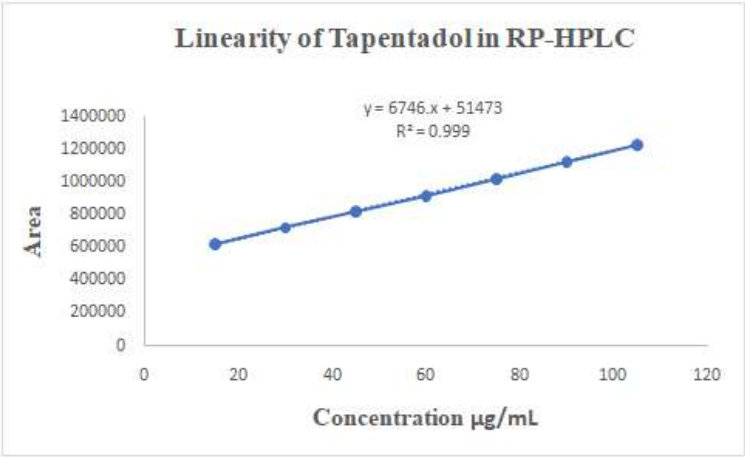

Fig. 3: Linearity plot of Tapentadol

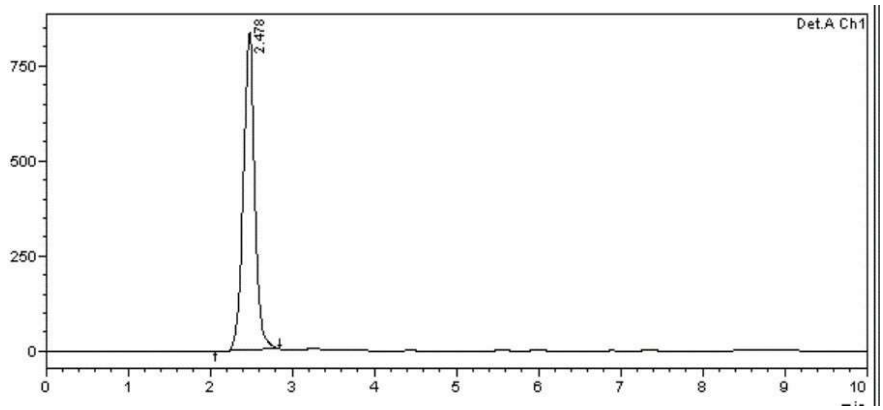

Fig. 4: HPLC Chromatogram of standard Tapentadol 60 µg/mL with retention time period of 2.478 minutes

lowest analyte level that may be accurately determined has been designated as the limit for quantitation. These parameters were computed with two equations: LOD = 3.3 × (σ/S) as well LOQ = 10 × (σ/S), wherein S is the average slope established by the calibration curve, having the standard variation from the intercept denoted as σ. The standard deviation has been calculated from six replicate determines of blank samples, and the slope was determined from the regression analysis utilizing the analyte's calibration plot. LOD as well LOQ values were expressed in terms of analyte concentration (16-20).

Robustness

A number of analytical parameters, including the composition of the mobile phase, rate of flow, detection wavelength as well retention time, RRT as well resolution, the total number of theoretical plates, along with column temperature, were purposefully unique in order to assess the robustness of advanced HPLC approach for Tapentadol. The optimal flow rate was partially varied from by the use of two alternative flow rates, 0.65 mL/min and 0.75 mL/min, to assess the impact of flow rate differences. The peak areas that resulted from six replicate injections at each flow rate were identified. Furthermore, adjustments to the UV

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Table 1: Linearity data of Tapentadol by HPLC

Concentration	Area	Retention Time	Theoretical Plate	Tailing Factor
15	617574	2.482	2172.437	1.125
30	718975	2.468	2178.487	1.128
45	819956	2.474	2161.437	1.126
60	913456	2.478	2178.487	1.121
75	1017956	2.481	2168.482	1.124
90	1119965	2.476	2172.594	1.126
105	1228746	2.473	2178.479	1.127

Table 2: Accuracy of tapentadol recovered

Percentage Level	Sample 60 µg/mL Area	Standard 15 µg/mL, 30 µg/mL, 45 µg/mL Area	Total Area	% Recovery	Mean % Recovery
50% (60 µg/mL + 15 µg/mL	904856	617574	1522325	99.98%	99.97%
			1522310	99.98%	
			1522283	99.97%	
100% (60 µg/mL + 30 µg/mL)	904856	718975	1623612	99.96%	99.92%
			1623543	99.95%	
			1622896	99.86%	
150% (60 µg/mL + 45 µg/mL)	904856	819956	1722465	99.71%	99.55%
			1721023	99.53%	
			1720056	99.41%	

*Accuracy data of RP-HPLC indicates triplicates n=3 for each standard and test sample three readings of same concentration and average of three were taken as per ICH Q2R2 guidelines

detection wavelength (λmax) were made to observe their influence on the chromatographic performance (21). The %RSD (Relative Standard Deviation) was calculated under each modified condition to determine the method's reliability and consistency, as summarized in (Tables 3 and 4).

Estimation of Tapentadol in Commercial Tablet Formulation

For the estimation of Tapentadol (TPD) in a marketed formulation, Tandol-100 tablets (containing 100 mg of TPD) were procured from a local pharmacy. A working solution was prepared by finely powdering twenty tablets using mortar along pestle. A 25 mL volumes flask had been added with exact-weighed powder that was similar to one tablet dose (25 mg, calculated from the average tablet weight). For optimal dissolution, 10 mL of the mobile phase has been added as well as the entire mixture was sonicated just for 20 minutes employing a bath sonicator. By adding the mobile phase to get the volume reaching 25 mL, the solution had been filtrated through a 0.45 µm sheets of membrane filter. This resulting in preparation of a 60ppm test solution within the desired linearity range. By contrasting the peak areas and also concentrations in the samples along with standard solutions, an assay % and sample chromatogram has been shown in (Fig. 5).

Preparation of reagents

0.01M Sodium Hydroxide Solution Preparation

40 milligrams were loaded in a 100 ml volume flask and diluted utilizing water and PH 9 was adjusted with dilute HCL.

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Table 3: HPLC Robustness data for tapentadol			
Robustness	Flow rate at 0.65 mL/min	Flow rate at 0.7 mL/min	Flow rate at 0.75 mL /min
Concentration	Area	Area	Area
60 µg/mL.	912456	913456	919864
60 µg/mL.	912345	915467	918665
60 µg/mL.	912786	915674	919567
60 µg/mL.	912668	917864	919597
60 µg/mL.	912678	915674	919653
60 µg/mL.	912567	917865	918573
Mean	912583.3333	916000	919319.8333
SD	161.5471036	1668.61008	553.4612603
%RSD	0.017702176%	0.182162673%	0.060203342%

Table 4: HPLC Robustness data for given parameters	
Wavelength Variation (±2 nm)	
272 nm	915874
274 nm	917456
276 nm	916235
Mean Area	916521.6
%RSD	0.086%
pH Variation (±0.2 units) Mobile phase pH area	
3.3	914876
3.5	915342
3.7	914965
Mean Area	915061
%RSD	0.025%
Column Temperature Variation (±5°C) Temperature (°C) Area	
25	915243
30	915874
35	916004
Mean Area	915707
%RSD	0.041%
Mobile Phase Composition (±2% Organic Content)	
Methanol	Area
48%	914678
50%	915874
52%	914965
Mean Area	915172
%RSD	0.063%

Preparation of Gibbs reagent

Weighed 100 mg of Gibbs Reagent in 50 ml volume flask, then added ethanol to dilute it completely.

Preparing of stock solution (1000 ppm) and working standards

Weighed 0.025 g of Tapentadol pure drug in 25ml volumetric flask and dissolve and makeup with water. In a 10-milliliter volumetric flask, pipette out 0.8 milliliters of the standard solution, then filled it with water for 80ppm.

Bioanalytical Extraction procedure and Recovery Study

To evaluate the recovery of Tapentadol from human plasma, a simple protein precipitation extraction method was employed. Briefly, 1 mL of human plasma was mixed with 1 mL of methanol and 1 mL of Tapentadol stock solution (60 ppm) in a centrifuge tube. The mixture was vortexed for 2 minutes to ensure thorough mixing and protein precipitation. Following this, the sample was centrifuged at 3000 rpm for 15 minutes. The clear supernatant was carefully collected and transferred into a 10 mL volumetric flask, diluted to volume with distilled water, and analyzed against a reagent blank using UV-Visible spectrophotometry at 653 nm.

The percentage recovery of Tapentadol from plasma was found to be

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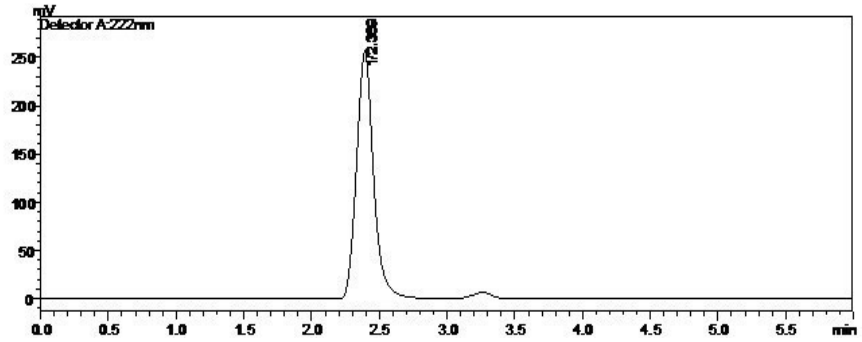

Fig. 5: Chromatogram of dosage form 60 µg/mL with retention time period of 2.478 minutes

98.45%, indicating excellent extraction efficiency and suitability of the method for routine bioanalytical quantification.

Blank plasma Preparation

After measuring mL of plasma and transferring it to a 10mL volumetric flask, the reagents were added together with water.

Chromogenic Bioanalytical Method validation at 653nm by UV-Visible Spectroscopy following M10 Guidelines

LLOQ (Lower Limit of Quantification, 20 µg/mL): The lowest concentration of the analyte that can be quantified reliably with acceptable accuracy and precision.

LQC (Low Quality Control, 60 µg/mL): A QC sample at the lower end of the calibration range, used to assess method performance near the LLOQ.

MQC (Medium Quality Control, 120 µg/mL): A QC sample near the midpoint of the calibration range, representing typical concentrations expected in samples.

HQC (High Quality Control, 200 µg/mL): A QC sample at the upper end of the calibration range, used to verify accuracy and precision at high concentrations.

ULOQ (Upper Limit of Quantification, 240 µg/mL): The highest concentration of the analyte that can be quantified reliably with acceptable accuracy and precision.

The quality control (QC) concentrations were selected based on the calibration range of 20–240 µg/mL in accordance with ICH M10 guidance. The LLOQ was set at 20 µg/mL to represent the lowest reliably quantifiable concentration. Low QC (LQC, 60 µg/mL) was chosen near 3× LLOQ to assess performance at the lower end, medium QC (MQC, 120 µg/mL) at the midpoint of the calibration range, and high QC (HQC, 200 µg/mL) near the upper range. The ULOQ was 240 µg/mL, representing the maximum calibrated concentration. These levels ensure the method is validated across the full analytical range for accuracy and precision.

Specificity

The abilities of a bioanalytical approach to differentiate among and recognize products composed of many different substances, including related molecules, is referred to as specificity. At the LLOQ, interfering substances shouldn't account for over 20% of each analyte response.

Selectivity

The capacity of the bioanalytical technique to distinguish and measure the analyte when the sample contains other components Known as selectivity. Selectivity was performed by scanning blank plasma.

Procedure for preparation of LLOQ and HLOQ samples

The plasma sample had been pipetted into two separate test tubes, and 2

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mL of 200 µg/mL TPD from 1000 µg/ml had been added. After that, 1 mL of methanol was put into each test tube. Vortexing the solution took two minutes. The contents were vortexed before being placed in a centrifugation tube and centrifuged for 15 minutes at 3000 rpm. It was gathered along the supernatant phase in a 10 ml capacity flask. A pH 9 solution containing sodium hydroxide in 1 mL has been combined with 0.2% Gibbs reagent. Water has been added. UV-visible spectroscopy was used to evaluate the LLOQ sample at 653 nm against a reagent blank.

Linearity

The calibration curve shows how an analyte's actual concentration and the assessment platform's response to it are related. When a specific amount of analyte or analytes are combined into a matrix, the calibration standards are formed, which comprise calibration curve. Calibration standards also need to be made using the same biological matrix that was used to make the study samples (22, 23). The LLOQ calibrating reference must fall within ±20% of the actual concentration, and at all other levels, it should be within ±15%.

Accuracy and Precision

Same prepared sample runs and data used to evaluate accuracy and precision for Precision there are two levels within run and between run for within run n=5 replicates were taken and for between run n=3 replicates were taken for each QC samples. Between run precision should not be greater than ±15% of any concentration level. Concentration's accuracy (%CV) at any given level cannot go above 15%. The (%CV) of LLOQ should not be greater than 20% as mentioned in ICH M10 guidelines.

Matrix effect

A matrix effect occurs when intervening and occasionally invisible substances within the sample matrix alter the analyte response. At least three distinct samples of LQC and HQCs should be examined to evaluate the matrix influence, with each generated matrix originating from a minimum of six different sources. The matrix effect should be assessed by looking at a minimum of three copies of LQCs and HQCs, each made using a matrix from a minimum of six different sources or lots. Regarding each matrix. The precision (also known as the percent coefficient of variation, abbreviated %CV) must not be beyond 15%, and the accuracy should fall around ±15% of the nominal concentration. (24,25)

Stability

Stability studies are required to make sure that choices regarding the handling of samples as well processing, evaluation, as well storage conditions don't affect the analyte's concentration. At both extreme and low concentrations, the material's endurance in the matrix is evaluated using QCs.

Analysis had been done on the LQC then HQC aliquots, and the appropriate storage conditions were implemented. The bulk sample must be divided into a minimum of three aliquots for every concentration under analysis. These samples will next go through analysis, storage, and filtering. (26-28).

Bench-top (short-term) stability

Only plasma samples were stored at 4-10°C then taken on day of analysis and spiked them with standard and reagents for conducting bench top stability studies

In stability studies only benchtop studies were conducted and %RSD was reported. It is essential to plan and carry out benchtop matrix stability studies that consider the handling circumstances of the study materials in the lab. Thawing high and low QCs on the tabletop should be done at the same temperature and for the same length of time as the study materials**.**

Reinjection reproducibility

To assess injection repeatability after storage, a run comprising at least five

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duplicates of each medium, low, and high QC along with calibration standards is reinjected. The continued existence accuracy of the samples handled is established along correctness of the reinjected QCs.

Results and Discussion

RP-HPLC Chromatographic Method

Selectivity

For Tapentadol, the RP-HPLC calibration curve (15–105 µg/mL) showed linearity with Y=6746.4X+514733; R^2=0.9997, and the chromogenic bioanalytical method (20–240 µg/mL) was linear with Y = 0.0051X + 0.0916; R^2=0.9996. 95% confidence intervals for the slope and intercept were calculated to demonstrate reliability:

RP-HPLC: slope 6746.4 (95% CI: 6700–6792), intercept 514733 (95% CI: 510000–519466)

Chromogenic method: slope 0.0051 (95% CI: 0.00495–0.00525), intercept 0.0916 (95% CI: 0.088–0.095). These intervals confirm that both methods are statistically robust and reliable for quantitative analysis of Tapentadol.

Linearity

RP-HPLC the concentrations of 15-105 µg/mL was linear coefficient of y = 6746.4x + 514733
R^2 = 0.9997.

Precision

Interday along with Intraday precision

It was determined that Tapentadol's %RSD of repeatability was 0.182162673%. The intra-day precision percentage RSD for the morning period was 0.242581892%, while the inter-day precision percentage RSD for both the first and second days was 0.242581892% and 0.111773988%, respectively. The evening's result was 0.160291444%. Every result met the ICH requirements to fall within limits.

Accuracy

Limits of detection and quantification

Both LOD as well as LOQ have been determined by formulas dependent on the calibration curve's slope as well standard deviation of the intercept (y) of regression lines, and the outcomes are 0.8162 µg/mL and 2.4733 µg/mL, respectively.
Standard Deviation =1668.61008
Slope =6746.4

Limit of Detection (LOD) =

$$\frac{3.3\times 1668.61008}{6746.4}=0.8162\ \mu g/ml$$

Limit of Quantification (LOQ) =

$$\frac{10\times 1668.61008}{6746.4}=2.4733\ \mu g/ml$$

Robustness

Robustness assessment was carried out assuming flow rate at -1(%RSD was 0.017702176%) and +1(%RSD has been 0.060203342%). The results obtained are within acceptable bounds and satisfied ICH requirements.

The developed method is robust against small, deliberate changes in experimental conditions such as flow rate, wavelength, pH, column temperature, and mobile phase composition, with %RSD values consistently below 2%, in accordance with ICH Q2(R2) guidelines.

Assay of marketed formulation

$$\%Assay=\frac{\text{Sample area}}{\text{Standard area}}\times\frac{\text{Standard Concentration}}{\text{Sample Concentration}}\times 100$$

$$\%Assay=\frac{904856}{913456}\times\frac{60}{60}\times 100$$

% Assay=99.05%

Chromogenic Bioanalytical Method validation at 653nm by UV-Visible Spectroscopy following M10 Guidelines

Validation parameters

Specificity

To ensure specificity of the developed Bioanalytical UV-visible

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spectrophotometric method, blank plasma from six sources was tested and showed no absorbance at the selected λmax. Spectral comparison confirmed that Tapentadol has a distinct absorbance maximum, with no interference from known metabolites. Additionally, the protein precipitation method likely eliminates polar conjugated metabolites such as glucuronides. These findings confirm the method's specificity for Tapentadol in plasma.

Selectivity

Selectivity was performed by scanning blank plasma in UV- Visible spectroscopy.

Linearity -Methodology to develop a calibration curve

The standard TPD drug solution was aliquoted into a variety of 10 ml volume flasks of varied proportions 0.2, 0.5, 0.6, 0.8, 1.0, 1.2, 1.6, 2.0 and 2.4 mL. 1 mL plasma spike was added to each 10 mL vol flask. Additionally, they treated the above-spiked plasma with a pH 9 NaOH solution and 0.2% Gibbs reagent. Blue-coloured chromogen of TPD absorbance at 653 nm was examined in relation to the reagent blank. Considering multiple concentrations of 20, 50, 60, 80, 100, 120, 160, 200, and 240 µg/mL, the linearity of the calibration curve (absorbance vs. concentration) for TPD examined in pure solution. Mean ± standard deviation for the slope, intercept, and correlation coefficient was determined for each of the six standard curves as shown in (Table 5 and Figs. 6, 7).

Accuracy and Precision

Preparation of Precision samples

0.2, 0.5, 0.6, 0.8, 1.0, 1.2, 1.6, 2.0 and 2.4 mL aliquots of the TPD standard drug solutions have been added to a series of 10 mL volumes flask. Each 10 mL volume flask has been diluted with 1 mL of plasma. Centrifugation provided the drug solution. Reagents were introduced after the supernatant phase was collected and volume had been adjusted through adding water. The final solution was checked with the appropriate reagent blank at 653 nm employing UV-visible spectroscopy. Five QC samples had been assessed to establish their mean ± standard deviation as shown in (Table 6). Between-run and within run %RSD or %CV for LLOQ levels exceeds 15% as shown in (Table 6) this meets ICH M10 criteria as it should be within limits of not greater than 20%. For this LLOQ samples average, standard deviation and %RSD was calculated.

Table 5: Calibration range of TPD with Gibbs reagent

Concentrations	Absorbance
20 µg/mL	0.2034
50 µg/mL	0.3345
60 µg/mL	0.4012
80 µg/mL	0.5012
100 µg/mL	0.6132
120 µg/mL	0.7012
160 µg/mL	0.9210
200 µg/mL	1.1234
240 µg/mL	1.3214

Matrix effect

Procedure for preparation of LQC, MQC & HQC Samples with different matrices

Preparation of LQC (60 µg/mL), HQC (200 µg/mL)

0.6 ml and 2.0ml of standard TPD solution and separate 1 ml of plasma have been pipetted onto a different 10 ml volume flasks; the solution was collected following centrifugation. Reagents have been added after the supernatant phase had been extracted and three separate runs were made. Water was added to make up the difference in concentration for the solution. The final solution of 60ppm and 200ppm has been examined over a corresponding blank reagent at 653 nm utilising UV-visible spectroscopy as shown in (Table 7) all the LQC and HQC samples were found to be within limits as per ICH M10 guidelines %CV should not exceed 15%.

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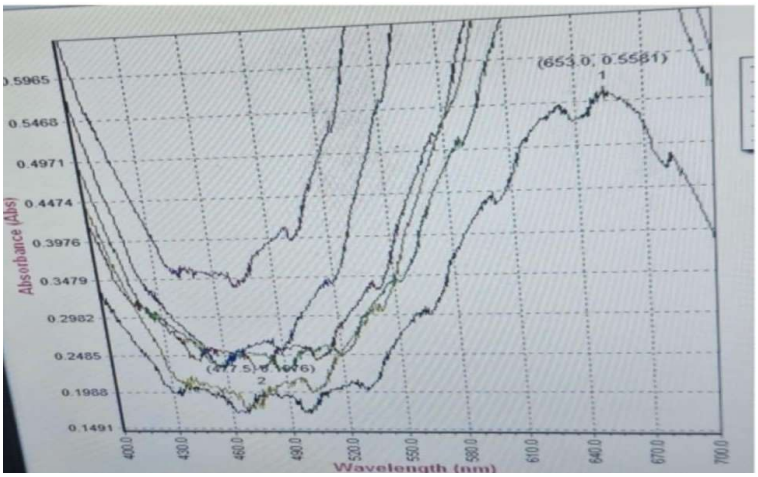

Fig. 6: Overlay mode spectrum of tapentadol at 653 nm

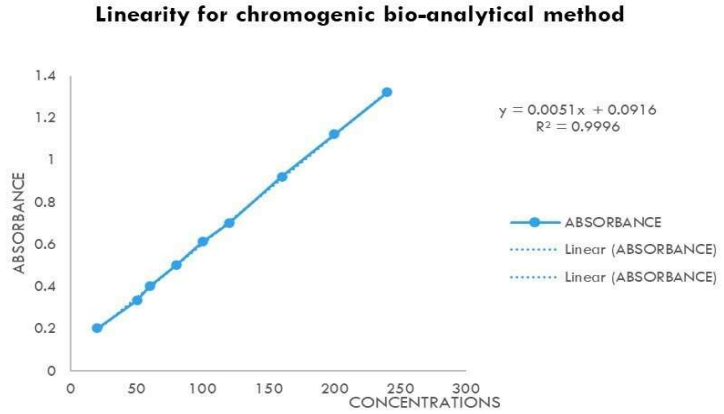

Fig. 7: Chromogenic bioanalytical calibration plot of TPD

Bench top stability studies

Procedure

QC sample preparation for LQC (60 µg/ml) along with HQC (200 µg/ml)

0.6 mL and 2.0 mL of the standard drug solution (1000 µg/mL) are inserted into two individual 10 mL vol flasks which had already been spiked with 1mL plasma. After gathering the supernatant layer, reagents were introduced. Two different concentration replicas were produced and stored at -20°C in a freezer for a longer duration as shown in (Table 8). The final solution of 60 and 200 µg/mL has been examined over a corresponding blank reagent at 653 nm utilising UV-visible spectroscopy.

Reinjection reproducibility

Procedure

Preparation of LQC 60 µg/ml, MQC 120 µg/ml and HQC 200 µg/ml samples

Stock sol (1000 µg/mL) was added to 3 different 10 mL volumes of flasks that had already been spiked with plasma using 0.6 mL,

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Table 6: Between-run and Within-run precision data of tapentadol					
Between-run precision data of tapentadol for the day – 1					
Between-run (Day-1)	20µg/mL (LLOQ)	60µg/mL (LQC)	120µg/mL (MQC)	200µg/mL (HQC)	240µg/mL (ULOQ)
Average	0.2931	0.4310	0.7323	1.132933	1.32657
SD	0.0111547	0.01215372	0.0223456	0.02187654	0.0123875
%RSD	17.15%	11.13%	12.23%	10.27%	14.15%
Between-run precision data of tapentadol for the day – 2					
Between run (Day-2)	20µg/mL (LLOQ)	60µg/mL (LQC)	120µg/mL (MQC)	200µg/mL (HQC)	240µg/mL (ULOQ)
Average	0.241166667	0.447866667	0.733366667	1.11363333	1.33657
SD	0.02122202	0.036735231	0.121563117	0.10746481	0.0223875
%RSD	18.26%	10.93%	14.21%	11.21%	13.12%
Within-run precision data of Tapentadol for each run					
Within run	20µg/mL (LLOQ)	60µg/mL (LQC)	120µg/mL (MQC)	200µg/mL (HQC)	240µg/mL (ULOQ)
Average	0.29361667	0.455233333	0.77645	1.197666667	1.35578
SD	0.0314006	0.043100108	0.073024401	0.114569756	0.0123875
%RSD	20.97%	13.87%	12.41%	14.37%	14.17%
*For Bioanalytical methods Precision there are two levels within run and between run for within run n=5 replicates each concentration prepared 5 times average was taken and for between run n=3 replicates each concentration prepared 3 times and average was taken for each QC samples as per ICH M10 guidelines					

Table 7: Tapentadol Matrix effect with LQC & HQC samples				
QC Concentration Levels	Matrices	Mean	SD	% CV
LQC 60 µg/mL	Matrices 1	0.447866667	0.036735231	10.93%
	Matrices 2	0.455233333	0.043100108	13.87%
	Matrices 3	0.431	0.01215372	11.13%
	Matrices 4	0.4478	0.04458754	13.98%
	Matrices 5	0.4567	0.04678321	14.09%
	Matrices 6	0.445	0.039812	11.65%
HQC 200 µg/mL	Matrices 1	1.132933	0.02187654	10.27%
	Matrices 2	1.11363333	0.10746481	11.21%
	Matrices 3	1.197666667	0.114569756	14.37%
	Matrices 4	1.112933	0.036421	11.28%
	Matrices 5	1.114563333	0.10953214	12.34%
	Matrices 6	1.187666667	0.1096543	14.83%

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Table 8: Tapentadol stability investigation with LQC & HQC				
QC Concentration Levels	Freeze Thaw Cycle	Mean	SD	% CV
LQC 60 µg/mL	1st cycle	0.44767	0.036735231	10.93%
	2nd cycle	0.4552	0.04310013	12.87%
	3rd cycle	0.431	0.01215372	11.13%
HQC 200 µg/mL	1st cycle	1.112933	0.046421	11.28%
	2nd cycle	1.11456	0.10953014	12.34%
	3rd cycle	1.189667	0.1096593	14.83%
In stability studies only benchtop studies were conducted and %CV for LQC and HQC samples n=3 was found to be within limits that is less than 15% as per ICH M10 guidelines.				

Table 9: Reinjection reproducibility data of Tapentadol				
QC Levels	Concentrations	Average	SD	% CV
LQC	60 µg/mL	0.4487	0.004408656	12.20%
MQC	120 µg/mL	0.7368	0.007230987	11.13%
HQC	200 µg/mL	1.11298	0.1012823	14.11%
*It indicates 5 replicates as average of 5 replicates absorbance were taken from concentrations of 60, 120, 200 µg/mL standard deviation and %CV were calculated for reinjection reproducibility				

1.2 mL, and 2.0 mL solutions. After extracting the subsequent layer of supernatant, reagents had added. Water had been added for the remaining capacity to make appropriate level. A similar reagent blank was scanned against the final mixture at 653 nm using UV-visible spectroscopy as shown in (Table 9). To determine whether there were any variations in the sample that had been prepared for analysis, the sample again reinjected.

Discussion

The developed RP-HPLC method for Tapentadol is reliable, economical, and ideal for routine quality control. Unlike LC-MS/MS techniques, it does not require high-end instruments or complex procedures. Although LC-MS/MS offers greater sensitivity (ng/mL), this method achieves adequate detection limits (µg/mL) for dosage forms. It eliminates the need for internal standards and reduces analysis time. In comparison to other HPLC approaches, it employs a straightforward isocratic mobile phase. This simplifies the procedure, minimizes baseline disturbances, and extends column life. The method demonstrated strong robustness against minor changes in flow rate, pH, and temperature. Precision and accuracy were well within ICH Q2(R2) specifications. Its short run time (~6 minutes) allows for faster sample processing. Overall, the method offers a strong combination of efficiency, accuracy, and user-friendliness for Tapentadol analysis.

Conclusion

A reliable and efficient the RP-HPLC technique to enable the quantitative assessment of tapentadol has been effectively developed as well validated. The developed method was found to be precise, accurate, and consistent. It exhibited good linearity throughout the analyzed concentration range. It met all validation parameters, including system suitability, specificity, and robustness, following the ICH recommendations. This makes the approach appropriate for regular Tapentadol analysis along with quality control in both raw materials and finished medications. Validated

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chromogenic UV Visible spectrophotometric approach for quantitative estimation of Tapentadol in biological matrices have proven to be accurate as well precise, and reliable. Although LC–MS/MS offers superior sensitivity and selectivity, it requires expensive instruments and complex sample preparation. The developed UV–Visible method with MBTH provides a simpler, cost-effective, and rapid alternative with adequate accuracy and precision, making it suitable for routine quality control despite slightly lower sensitivity. The successful validation of these methods confirms their efficacy and detection of Tapentadol in biological samples.

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Conflict of Interest

The authors report that there are no competing interests to declare.

Abbreviations

TPD: Tapentadol; SD: Standard deviation; UV: Ultraviolet; % RSD: Relative standard deviation; SGT: Sitagliptin; LLOQ: Lower limit of quantification; LOQ: Limit of quantitation; CV: Coefficient of variance; LOD: Limit of detection; ICH: International Council on Harmonization; PPM: Parts per million; RP: Reverse phase; µg: Microgram; ULOQ: Upper limit of quantification; Mg: Milligram; mL: Milliliters; %: Percentage; HPLC: High-performance liquid chromatography.

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