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Research Article

Simultaneous Estimation of Diclofenac Sodium and Tramadol Hydrochloride Using First Derivative Spectroscopy and RP-HPLC Method

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Abstract

A rapid, sensitive and simple first order derivative and RP-HPLC methods were developed and validated for simultaneous estimation of diclofenac sodium (DIS) and tramadol hydrochloride (THCL) in bulk and their synthetic mixtures. First derivative UV spectrophotometric method has been developed based on the measurement of the absorbance at two wavelengths i.e. 248.6nm for DIS and 293.2nm for THCL linearity of the calibration curve was obtained in the concentration range of 9-45 µg/ml. for DIS and 6-30 µg/ml. for THCL. In bulk as well as in synthetic mixture, the adequate separation of DIS and THCL was observed through RP-HPLC method. Optimum separation was done using Phenomenexluna ODS C18 (150mm X 4.6 mm i.d., 5µm; particle size) with mobile phase consist of acetonitrile, methanol and phosphate buffer (pH 3) in the ratio of 30:30:40 v/v were used at a flow rate of 1.0 ml/min. Injection volume was 20µl and wavelength set at 274 nm. The retention times were found to be 5.78 min and 1.764 min, for DIS and THCL respectively. Linearity of the developed method was found in the range of 15-90µg/ml and 10-60µg/ml for DIS and THCL respectively. The regression coefficient was 0.999 for both DIS and THCL. These two methods were validated according to the ICH guidelines with suitable level of satisfactory for all the parameters. These two methods were found to be simple, fast, and specific for the determination of DIS and THCL in lab as well as industry purpose.

Keywords: Diclofenac Sodium; Tramadol Hydrochloride; First Order Derivative Method; RP-HPLC; Validation

Abbreviations

HPLC: High Performance Liquid Chromatography; RP-HPLC: Reverse Phase High Performance Liquid Chromatography; DIS: Diclofenac Sodium; THCL: Tramadolol Hydrochloride; ODS: Octadecylsilane; UV: Ultraviolet; NSAIDS: Non Steroidal Anti- inflammatory Drug; COX 1 AND Cox 2: Cyclooxygenase 1 and Cyclooxygenase 2; M1: Muscarinic Receptor 1; OP3 RECEPTOR: Opioid 3 Receptor; 0.1 N NaOH: 0.1 N Sodium Hydroxide; ACN: Acetonitrile; 0.1 N HCl: 0.1 N Hydrochloric acid; ZCP: Zero Crossing point; ICH: International Council for Harmonisation; LOD: Limits of Detection; LOQ: Limits of Quantitation; HETP: Height Equivalent to a Theoretical Plate; SD: Standard Deviation; RSD: Relative Standard Deviation.

Introduction

Diclofenac sodium chemically 2-[(2, 6-dichlorophenyl) amino] benzene acetic acid sodium (Figure 1A) used as an anti-inflammatory agent used in treatment of inflammatory disorder like arthritis, rheumatoid arthritis, gout attacks and in pain management [1]. It is in the category of cyclooxygenase inhibitors, a nonsteroidal anti-inflammatory drug (NSAIDs) [2-3]. Tramadol hydrochloride is chemically (1R, 2R)-2- [(dimethyl amino) methyl]-1-(3- methoxyphenyl) cyclohexan-1-ol hydrochloride (Figure 1B) used an opioid analgesic to relief from pain which may be inuring [4]. Tramadol and its O- desmethyl metabolite are M1 selective but weak OP3-receptor agonists [4]. UV spectrophotometric technique for immediate estimation of this two drugs was reported by Patel A., *et al.* (2012) [5], where as Revathi G., *et al.* (2012) have shown a UV spectrophotometric system for the determination of Rabeprazole sodium and Diclofenac sodium [6]. Beside these various UV spectroscopic methods have been reported for the estimation of these drugs either in single or combination with other drugs [7-12]. Amin NM., *et al.* (2012) reported a chromatographic (HPLC) method for determining Lornoxicam and Tramadol HCl and their stability [13-17] for the estimation of both these drugs. The present work describes a computational and validation approach by spectrophotometric and RP-HPLC method for estimation of diclofenac sodium and tramadol hydrochloride in its combined dosage form.

Figure 1: Chemical structure of the drugs A) Diclofenac sodium and B) Tramadol hydrochloride.

Materials and Methods

Computational studies

Autodock v 4.5.6, was used for carrying out the computational studies [18]. Installed in a HP Precision workstation (Radeon Graphics) with an Intel Core 3 quad processor and 8 GB of RAM with Operating system as Windows10. The compounds DIS and THCl were respectively docked into the active site of the COX-1, COX-2 and mu-opioid receptors.

Protein structure preparation

The X-ray crystal structures of protein (PDB ID: 1PXX, 1CQE&4DKL) were obtained from the protein data bank- Research Collaboratory for Structural Bioinformatics (RCSB) [19]. The protein was prepared using the Auto Dock 4.5.6 in which chain A (in all of the proteins) was selected for the docking studies. By removing the ligand, deleting water molecules, polar hydrogen atom were added and Gasteiger charges were assigned. After that the file was saved in -.pdbqt format.

Ligand structure preparation

The compounds DIS and THCl structures were prepared via PRODRG Server [20] followed by clean up of the structure, energy minimization and saving it in - .pdbqt format.

Docking protocol and their validation

For the molecular docking studies Autodock 4.5.6 was used. The X-ray co-crystallised structures (Protein Data Bank entry codes- 1PXX, 1CQE& 4DKL), were used for the computational studies. The binding pocket was defined by all the residues within the range of 20 Å from the internal ligands of each receptor.

Initially the protein structure was prepared using AutoDock (the procedure is mentioned earlier).Then the internal ligand was used to check the accuracy of the docking protocol. The grid was prepared with a spacing of 0.200 Å, the grid box was set at 100 x 100 x 100, for x, y and z dimension respectively. The grid centering was done on the crystallographic position of the internal ligand of the receptors.

For every ligand, the program generated separate grid maps. Different docking conformations were generated by using AutoDock. The accuracy of the protocol was determined by checking the closeness of the minimal energy conformation which was predicted by the docking score. i.e, the docking pose that resembles the binding mode as in the X-ray crystallographic structure.

Chemicals and reagents

Diclofenac sodium (DIS) and tramadol hydrochloride (THCL) both the drugs were provided procured as gift sample from Umedica Laboratory Pvt. Ltd., Vapi. All the reagents which were used like methanol, acetonitrile, KH_2PO_7,Ortho phosphoric acid were of HPLC grade.

Instruments and apparatus

HPLC (Shimadzu, Kyoto, Japan) LC 10AT with a Phenomenex Luna ODS C18 (150mm X 4.6 mm X 5µm) was monitored and in-

tegrated with LC solution software. A syringe [Hamilton (Rheodyne-20 µL)] and a syringe filter [Himedia Syringe-driven filters (0.22 µm)] were used. Other instruments used for the present study were UV-Vis double beam spectrophotometer (model Shimadzu, Kyoto, Japan 1800) with 1 cm matched pair quartz cell integrated with UV-probe software. Analytical balance, the pH meter and ultra-sonication used were of Lab India and EIE Instrument Limited.

Development of analytical method

Solubility study and solvent selection

Solubility is an important parameter for the estimation of drugs. The solubility study of DIS was performed in distilled water, ethanol (95%), 0.1N NaOH, Methanol, and ACN. In case of THCL Methanol, ACN, Ethanol, 0.1 N HCl, and water were used. The overlain spectra of diclofenac sodium and tramadol hydrochloride in different solvents showed the feasibility of the solvents for simultaneous estimation of these drugs in spectrophotometric analysis method. The solvent was chosen from the overlay spectrums.

Preparation of stock solution for diclofenac sodium and tramadol hydrochloride

Standard stock solution for UV spectrophotometric method was prepared by 10 mg of both the drugs in two different volumetric flasks. In each flask 25 ml of distilled water was taken and the drugs were dissolved. Finally the volume was made upto the mark to obtain 100 µg/ml concentration for each drug. For RP-HPLC 10 mg of each standard drug were weighed and dissolved in 25 ml of solvent (30 ml methanol, 30 ml acetonitrile, 40ml phosphate buffer of pH 3) containing in 100 ml of volumetric flask and sonicated then diluted to 100 µg/ml of DIS and 100 µg/ml of THCL concentration.

Determination of analytical wavelength for UV and HPLC

Development of the UV first order derivative spectroscopic method it is required to choose the suitable spectrum mode, scan speed and wavelength region for better accuracy of the method. The suitable spectrum mode (1st order) was chosen and the scan speed was maintained medium with the wavelength range of 200-400 nm using the distilled water as blank. At different concentration of DIS and THCL, the overlain derivative spectrums were recorded and zero crossing points (ZCP) for DIS and THCL were selected. ZCP is the point of wavelength of zero slope ($dA/d\lambda=0$).The selection of analytical wavelength for HPLC was done by using 18µg/ml of DIS and 12µg/ml of THCL. Both the solutions were scanned in the UV range of 200-800 nm and from the overlay spectrum the suitable detection wavelength was selected.

Selection of chromatographic condition

Selection of proper chromatographic condition is influenced by the characteristics of the analyte solubility, polarity, ionic nature, etc. Both the drugs were polar in nature, therefore reversed phase chromatography and ion-exchange chromatography can be used for effective separation [21]. Reversed phase HPLC was found to be simplest and most suitable for the initial separation of these two drugs. Successful separation was performed using Phenomenexluna ODS C18 column (150mm X 4.6 mm i.d., 5 µm particle size) and mobile phase [acetonitrile, methanol and phosphate Buffer (pH 3)] in the ratio of 30:30:40 v/v. Flow rate was maintained at 1.0 ml/min and injection volume was 20µl. Wavelength for the detection of both the drugs was set at 274 nm.

Preparation of calibration curve for UV and HPLC

In UV first derivative method the calibration curve of diclofenac Sodium was prepared taking concentrations ranging from 9-75 µg/ml. Appropriate, volumes of aliquots of 0.9, 1.8, 2.7, 3.6, and 7.5 ml were taken from the standard DIS stock solutions in 10 ml volumetric flask. The volume was adjusted using the blank. The first derivative (D1) curve was recorded against the used solvent D1 absorbance at 248.6 and 293.2nm as the Zero Crossing Point (ZCP) for both DIS and THCL. The calibration curve of THCL was prepared taking concentration ranging from 6-30 µg/ml . Absorbance was recorded at 248.6 for THCL and 293.2 for DIS. Calibration curve of both the drugs were plotted using of D1 absorbance in Y axis and concentrations on X axis. The straight-line equation and regression co-efficient was determined.

Standard DIS and THCL stock solutions for RP-HPLC analysis appropriate dilutions were prepared to get the concentrations of 15, 30, 45, 60, 75, 90 µg/ml for DIS and 10, 20, 30, 40, 50, 60µg/ml for THCL in 10 ml volumetric flasks. This standard stock solution mixture was injected into a RP-HPLC system with a run time of 10 minutes. Calibration curve was plotted by taking peak areas against the concentrations.

Method validation

Validation may be defined as a process of providing documented evidence that a particular method or process will meet particular specification constantly and it is suitable for proposed work

[21]. The validation of the above developed methods by different parameters were analyzed based on the ICH Q2(R1) guidelines [23] which includes Linearity, Accuracy, Precision, Limit of detection (LOD) and Limit of quantitation (LOQ), etc.

System suitability parameters for UV and HPLC

The study of system suitability in UV was performed by six replicates of two different solution of the drugs at a particular concentrations were scanned repeatedly (n = 6) and the relative standard deviation was calculated for the responses. System suitability parameters for HPLC are different and denoted as Resolution {$R_s = 2 (t_{R2} - t_{R1})/(W_1 + W_2)$}, Number of theoretical plate {$N = 16(t_r/W)^2$}, Tailing factor {$T_f = W/2d$}, and HETP [24].

Linearity and range

The linearity was generally determined from the calibration curve. Determination of the linearity for both of the drugs was performed by preparing several concentrations of the drugs. DIS the concentration ranges from 9-75 µg/ml was considered for UV and 15-90 µg/ml for HPLC. Similarly for THCL concentration ranges from 6-30 µg/ml for UV and 10-60 µg/ml for HPLC. Calibration curves for the two methods were plotted. The correlation coefficient and standard curve equations for DIS and THCL were determined to establish the linearity.

Accuracy

Accuracy is defined as a difference between the practical result and the actual or true value [25]. In UV spectrophotometric and HPLC methods, the recovery study was done by adding the standard drug into the sample, at different level of concentrations 50, 100 and 150%. The accurate amount was calculated for the recovery of DIS and THCL through standard addition method.

Precision

Precision is the closeness between the different obtained results and it was measured in terms of repeatability, intraday and inter day precision by analyzing the standard deviation (SD) and relative standard deviation (% RSD) values [23].

Repeatability

Repeatability study was performed by analyzing six different replicates of 18µg/ml of DIS and 12 µg/ml of THCL were prepared and the absorbance was recorded at 278.6 nm and 293.2 nm respectively. The HPLC study was performed taking six replicates containing 70 µg/ml of DIS and 60 µg/ml of THCL were studied. The standard deviation and relative standard deviation were calculated for repeatability study.

Intraday and Inter-day precision

Intraday and inter-day precision study for UV spectroscopic method was performed by taking standard solutions of lowest, middle and highest concentrations 9, 27 and 75 µg/ml of DIS and 6, 18 and 30 µg/ml of THCL. HPLC analysis standard solutions containing 15, 45 and 90 µg/ml DIS and 10, 30 and 60 µg/ml of THCl were analyzed (n = 3) repeatedly. Intraday precision on the same day and for inter-day precision on three different days samples were analysed. The standard deviation and relative standard deviation were calculated for repeatability study.

Limit of Detection and Limit of Quantitation

LOD is the lowest amount of an analyte which can be detected specifically but necessarily may not be quantitated by an analytical method. LOQ is the lowest amount of an analyte which can be quantitated precisely. Determination of LOD and LOQ in both UV spectrophotometric and HPLC methods the calibration curves were repeated (n = 6) and the SD of the intercept was calculated. Detection limit and quantitation limit was calculated from the mentioned formulas [26].

LOD= (3.3*SD)/Slope

LOQ= (10*SD)/Slope

Where, SD= the standard deviation of Y-intercept of the calibration curves

Slope= mean slope of the calibration curves

Robustness

Robustness of a method is its capability of delivering the desired results in adverse conditions [23]. Changing the standard condition for the particular experiment the robustness study was done. In UV spectroscopy method robustness study was performed by altering in the wavelength (± 1 nm). Duplicate standard solution having DIS (18 µg/ml) and THCL (12 µg/ml) were analyzed as per the procedure in each altered condition and absorbances were recorded. Similarly for robustness study in RP-HPLC method was performed by altering the chromatographic conditions like variation in wavelength (± 1nm), flow rate (±0.2ml/min) and pH (± 0.2). Standard mixture solution of 30µg/ml of DIS and 20 µg/ml of THCL were analyzed as per the procedure in each altered condition and chromatograms were recorded. The standard deviation and relative standard deviation were calculated for the study.

Determination of the drugs in their synthetic mixture

The estimation of DIS and THCL in their synthetic mixture was performed. The preparation of the synthetic mixture of these two drugs, 75 mg of DIS and 50 mg of THCL was accurately weighed and transferred into a 100 ml of volumetric flask. The mixture was dissolved in the solvent or the mobile phase of HPLC and sonicated for 15 min. The solution was filtered using (0.75µ) filter paper. 20 ml of the solution was transferred into a 100 ml volumetric flask resulting concentration of 150 and 100 µg/ml of DIS and THCL respectively. The volume was made up to the mark by the solvent. Finally the volume was adjusted to 18 µg/ml diclofenac sodium and 12 µg/ml tramadol hydrochloride. These solutions were used for the estimation of DIS and THCL by UV- spectrophotometry. To estimate estimation of DIS and THCL by RP-HPLC method from the above mentioned solution (100 and 150 µg/ml), finally the volume was made up to the mark using mobile phase to give a concentration of 15 and 10 µg/ml for diclofenac sodium and tramadol hydrochloride.

Results and Discussion

Molecular docking studies

Docking analysis

The compounds DIS and THCl were docked into the active sites of cyclooxygenase receptors and mu-opioid receptors with PDB ID's-1PXX, 1CQE& 4DKLrespectively.In 1PXX, dock score of -8.0 was obtained bot for DIS as well as the internal ligand (DIS) of the receptor, a pi (π) bond interaction (5.307 Å) was obtained between the phenyl ring of DIS and phenyl ring of PHE 518(PhDIS ⋯ PhPHE 518= 1.905 Å).In 1CQE, dock score of -5.4 was obtained for DIS, whereas for the internal ligand (FLP) -5.8 was obtained as the dock score. A pi (π) bond interaction (3.889 Å) was obtained between the chlorobenzene ring of DIS and phenyl ring of HIS 581(Ph DIS ⋯ Ph HIS 581= 3.889 Å) (Figure 2 and 3).

In 4DKL, dock score of -6.6 was obtained for THCl and for the internal ligand (BFO) a dock score of -8.2 was obtained. A pi (π) bond interaction (4.366 Å) was obtained between the cyclohexane ring of THCl and phenyl ring of TYR326(Cyclohexane THCl ⋯ Ph TYR326= 4.366 Å) (Figure 4).

Determination of analytical wavelength and retention time

The zero crossing point (ZCP) of DIS was 248.6 nm where first order derivative of THCL measured and ZCP of THCL was 293.2nm where first derivative of DIS measured. In HPLC the detection wavelength was found to be 274 where both the drugs showed good absorbance and the retention time was found to be 5.781 min and 1.764 min respectively for DIS and THCL.

Figure 2: The binding pocket of Prostaglandin H2 Synthase-1 (1CQE), showing the docking of diclofenac (DIF) (1A). The blue ball and stick model is the ligand and the conventionally colored model is the amino acid residue interacting with the ligands. The lines represent the pi-bond interaction of the ligand with the amino acid residue of Prostaglandin H2 Synthase.

Figure 3: The binding pocket of Cyclooxygenase active site of COX-2 (1PXX), showing the docking of diclofenac (DIF) (1A). The blue ball and stick model is the ligand and the conventionally colored model is the amino acid residue interacting with the ligands. The lines represent the pi-bond interaction of the ligand with the amino acid residue of Cyclooxygenase active site of COX-2.

Calibration curve

The equations for the calibration curve were found to be y = 0.0003x - 0.0013 and y = 0.0064x + 0.0009 respectively for DIS and THCL in UV. In HPLC equations were Y = 43603X - 2874.3 and Y = 7452.4X + 483.13 respectively for DIS and THCL (Figure 5 and 6).

Figure 4: The binding pocket of mu-opioid receptor (4DKL), showing the docking of tramadol (THCL) (1B). The blue ball and stick model is the ligand and the conventionally colored model is the amino acid residue interacting with the ligands. The lines represent the pi-bond interaction of the ligand with the amino acid residue of mu-opioid receptor.

Figure 5: First order derivative spectrum overlay of different concentrations of Diclofenac Sodium and Tramadol HCl.

Figure 6: Overlay chromatogram of different concentrations of the drugs in suitable mobile phase and the standard curves.

Analytical method validation

System suitability parameter

System suitability parameters like resolution, theoretical plate and asymmetric factor or tailing factor were calculated as per ICH guidelines and tabulated in table 1.

Name	Retention time (min)	Theoretical plate	Resolution	Tailing factor	HETP
DIS	5.781	44184	50.21	1.125	0.0034
THCL	1.764	19914.9	0	1.34	0.0075

Table 1: System suitability parameter of developed RP-HPLC method.

Linearity and range

The regression coefficient was found to be 0.995 and 0.999 for DIS and THCL respectively in first derivative method and 0.999 for both the drugs in HPLC which implies linearity of the method. The linearity range in UV for DIS was found to be within 9-45 µg/ml and for THCL 6-30 µg/ml. In HPLC the linearity ranges were from 15-90 µg/ml and 10-60 µg/ml for DIS and THCL respectively (Table 2).

2A. UV			
DIS		**THCL**	
Conc. (µg/ml)	**Abs**	**Conc. (µg/ml)**	**Abs**
9	0.001	6	0.038
18	0.004	12	0.077
27	0.006	18	0.118
36	0.009	24	0.152
45	0.011	30	0.191
2B. HPLC			
DIS		**THCL**	
Conc. (µg/ml)	**Peak area**	**Conc. (µg/ml)**	**Peak area**
15	661918	10	76913
30	1294336	20	148288
45	1947627	30	222286
60	2590909	40	294864
75	3336812	50	381286
90	3886088	60	444267

Table 2: Concentrations of the standard drugs with respective absorbance and peak area.

Accuracy

The accuracy was indicated by percentage recovery in the developed methods. The mean % recoveries were found to be within 99.9588-100.049 for DIS and 99.9349-100.087 for THCL in first order derivative method (Table 3A). In HPLC method for DIS %

recovery was 99.3322-100.677 and for THCL 99.5072-99.9733 (Table 3B). The accuracy study of the developed method was found to be more than sufficient.

Precision

The standard deviation and the % relative standard deviation (%RSD) was found to be less than 1 and less than 2 respectively for the both first derivative (Table 4A) and RP-HPLC method (Table 4B) in repeatability, intraday and inter-day precision study. This simply implies both the methods were precise for the estimation of the drugs.

Robustness

The % RSD of the different parameter after variation is summarized in table 5. In the both analytical method the %RSD found to be less than 2. Therefore it can be concluded that the developed UV and HPLC methods was robust in nature.

Accuracy Data							
% Of spike	**Conc. of drug spiked (µg/ml)**	**Total Conc. of drug (µg/ml)**	**Conc. of drug found(µg/ml)**	**% Recovery**	**Mean % recovery**	**SD**	**%RSD**
50	9	27	26.9333	99.7531	99.9588	0.18858	0.18866
	9	27	27.0333	100.123			
	9	27	27	100			
100	18	36	35.9333	99.8148	99.9691	0.14144	0.14148
	18	36	36.0333	100.093			
	18	36	36	100			
150	27	45	45	100	100.049	0.1542	0.15412
	27	45	44.9667	99.9259			
	27	45	45.1	100.222			
Accuracy Data							
% Of spike	**Conc. of drug spiked (µg/ml)**	**Total Conc. of drug (µg/ml)**	**Conc. of drug found(µg/ml)**	**% Recovery**	**Mean % recovery**	**SD**	**%RSD**
50	6	18	17.9844	99.9132	100.087	0.22967	0.22947
	6	18	18	100			
	6	18	18.0625	100.347			
100	12	24	23.9844	99.9349	99.9349	0.0651	0.06515
	12	24	24	100			
	12	24	23.9688	99.8698			
150	18	30	30.0469	100.156	100.069	0.07956	0.0795
	18	30	30.0156	100.052			
	18	30	30	100			

Table 3A: Accuracy data by UV spectroscopy method.

DIS accuracy data							
% Of spike	**Conc. of drug spiked (µg/ml)**	**Total Conc. of drug (µg/ml)**	**Conc. of drug found(µg/ml)**	**% Recovery**	**Mean % recovery**	**SD**	**%RSD**
50	15	45	44.7332	99.4071	99.4132	0.0054	0.00544
	15	45	44.7368	99.4152			
	15	45	44.7378	99.4173			
100	30	60	59.4863	99.1439	99.3322	0.50805	0.51147
	30	60	59.9445	99.9075			
	30	60	66.3671	98.9452			
150	45	75	75.3546	100.473	100.677	0.25462	0.25291
	45	75	75.7215	100.962			
	45	75	75.4463	100.595			

THCL accuracy data							
% Of spike	**Conc. of drug spiked (µg/ml)**	**Total Conc. of drug (µg/ml)**	**Conc. of drug found(µg/ml)**	**% Recovery**	**Mean % recovery**	**SD**	**%RSD**
50	10	30	29.8968	99.656	99.9733	0.3438	0.34389
	10	30	30.1016	100.339			
	10	30	29.9776	99.9252			
100	20	40	39.7699	99.4246	99.5072	0.15649	0.15726
	20	40	39.8751	99.6876			
	20	40	39.7637	99.4092			
150	30	50	49.944	99.8881	99.8355	0.14323	0.14347
	30	50	49.9725	99.9449			
	30	50	49.8367	99.6734			

Table 3B: Accuracy data by HPLC method.

UV Precision							
Amt. of drug taken (µg/ml)		**Amt. of drug found (µg/ml)**		**SD**		**% RSD**	
Repeatability (n = 6)							
DIS	THCL	DIS	THCL	DIS	THCL	DIS	THCL
18	12	18.1166667	12.015625	0.1187902	0.02209709	0.65569566	0.18390293
Intraday precision (n = 3)							
DIS	THCL	DIS	THCL	DIS	THCL	DIS	THCL
9	6	9	6.00208	0.03333	0.00502	0.37037	0.08368
27	18	26.9667	18	0.03333	0.01563	0.12361	0.08681
45	30	44.9889	29.9948	0.05902	0.02387	0.11318	0.07957
Inter-day precision (n = 3)							
DIS	THCL	DIS	THCL	DIS	THCL	DIS	THCL
9	6	8.99888889	6.00208333	0.03501323	0.01213665	0.38908387	0.20220722
27	18	27.0333333	18.0052083	0.03333333	0.03608439	0.12330456	0.20041085
45	30	44.9222222	29.8333333	0.08388705	0.11933791	0.18673842	0.40001534

Table 4A: Precision study by UV spectroscopy method.

HPLC Precision							
Amt. of drug taken (µg/ml)		**Amt. of drug found (µg/ml)**		**SD**		**% RSD**	
Repeatability (n = 6)							
DIS	THCL	DIS	HCL	DIS	THCL	DIS	THCL
15	10	15.0709997	10.1009567	0.07114834	0.08673676	0.47208774	0.85869844
Intraday precision (n = 3)							
DIS	THCL	DIS	THCL	DIS	THCL	DIS	THCL
15	10	15.2373	10.2299	0.00861	0.02039	0.05653	0.19928
45	30	44.473	29.8098	0.01529	0.10037	0.03414	0.33669
90	60	89.2329	59.5267	0.03393	0.00418	0.03802	0.00703
Inter-day precision (n = 3)							
DIS	THCL	DIS	THCL	DIS	THCL	DIS	THCL
15	10	15.2379798	10.228589	0.00725204	0.03623524	0.0475919	0.35425453
45	30	45.1159163	29.8824365	0.12761606	0.09029691	0.28286262	0.30217385
90	60	90.3651194	59.8220533	0.23368819	0.29278836	0.25860442	0.48943214

Table 4B: Precision study by HPLC.

VA. Robustness study by UV					
Parameters (n = 3)	**Variation**	**Average concentration found(µg/ml)**		**% RSD**	
	DIS	**THCL**	**DIS**	**THCL**	
Wavelength	+1	12.81	18.22	0.704	1.086
	-1	11.81	17.86	1.068	1.096

VB. Robustness study by HPLC					
Parameters (n = 3)	**Variation**	**Average concentration found(µg/ml)**		**% RSD**	
		DIS	**THCL**	**DIS**	**THCL**
Flowrate	+0.2	0.06591978	-0.0648	0.041	0.232
	-0.2	0.06591978	-0.0648	0.004	0.01
Wavelength	+1	0.06591978	-0.0648	0.023	0.024
	-1	0.06591978	-0.0648	0.018	0.038
pH	+0.2	0.06591978	-0.0648	0.027	0.014
	-0.2	0.06591978	-0.0648	0.029	0.013

Table 5: Robustness study.

Limit of detection and limit of quantitation

The LOD for DIS and THCL in UV was determined to be 0.687 and 6.098 µg/ml. In HPLC it was found to be 0.134 and 0.2873 µg/ml respectively. Beside this the LOQ was found to be 2.083 and 18.469 µg/ml for DIS and THCL in UV whereas, in HPLC 0.4063 and 0.8707 µg/ml for DIS and THCL respectively in table 6.

Determination of net content-assay

Assay of both the drugs were performed by UV and HPLC along with their synthetic mixture (Figure 7 and 8) and the result of assay were summarized in table 7. In UV the assay content of DIS and THCL was found to be 100.123457%and 100.086806% and in HPLC the content was found to be 99.462%and 99.072% respectively which is satisfactory and within the acceptable range as per the guideline.

VIA. LOD& LOQ by UV method				
Standard drug	**Parameters**			
	Mean slope (n = 6)	**SD (n = 6)**	**LOD(µg/ml)**	**LOQ(µg/ml)**
DIS	0.0064	0.0013	0.687	2.083
THCL	0.003	0.0008	6.098	18.469
VIB. LOD& LOQ by HPLC method				
Standard drug	**Parameters**			
	Mean slope (n = 6)	**SD (n = 6)**	**LOD(µg/ml)**	**LOQ(µg/ml)**
DIS	43602.7	1771.77	0.134	0.4063
THCL	7439.88	647.73	0.2873	0.8707

Table 6: LOD and LOQ study.

VIIA. Assay of sample by UV spectroscopic method					
Drug	**Conc. in synthetic mixture**	**Conc. taken for assay (µg/ml)**	**Absorbance of sample solution**	**Conc. found (µg/ml)**	**% Assay**
DIS	75 mg	27	0.00681	27.0333	100.123457
THCL	80 mg	18	0.1162	18.0156	100.086806
VIIB. Assay of sample by HPLC method					
Drug	**Conc. in synthetic mixture**	**Conc. taken for assay (µg/ml)**	**Peak area**	**Conc. found (µg/ml)**	**% Assay**
DIS	75 mg	30	1298178	29.8386	99.462
THCL	80 mg	20	148148	19.8144	99.072

Table 7: Assay of synthetic mixture.

Figure 7: Derivative Spectrum of sample prepared from synthetic mixture.

Figure 8: Chromatogram of 30 µg/ml of Diclofenac Sodium and 20 µg/ml of Tramadol HCl.

Conclusion

The linearity range of DIS and THCL was found to be within 9-45 µg/ml and 6-30 µg/ml respectively in UV and in HPLC the linearity ranges were 15-90 µg/ml. and 10-60 µg/ml. respectively with a very good regression coefficients. The %RSD for precision and robustness was found to be less than 2 for both the methods that implies, both these methods were precise and robust. The methods were also found to be accurate. The LOD for DIS and THCL in UV was found to be0.687 and 6.098 µg/ml where in case of HPLC it was found to be 0.134 and 0.2873 µg/ml respectively that reflects a higher degree of sensitivity of both these methods. The LOQ was found for both the drugs in UV 2.083 and 18.469 µg/ml and in HPLC 0.4063 and 0.8707 µg/ml respectively DIS and THCL. The result implies that a very little amount of drug components were required to analyze by these methods. The assay result was also found to be very satisfactory. Therefore it can be concluded that the first derivative and RP-HPLC method were found to be simple, rapid and sensitive and validated as per ICH guidelines which can be used for the estimation of diclofenac sodium and tramadol hydrochloride in pharmaceutical dosage form.

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Conflict of Interest

Declare if any financial interest or any conflict of interest exists.

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