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Original Article

Development and Validation of Fast and Robust Stability Indicating RP-HPLC Method for Simultaneous Estimation of Tolperisone Hydrochloride and Etoricoxib in Pharmaceutical Dosage Form

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ABSTRACT

Objectives: A precise, accurate and selective stability-indicating reverse phase high performance liquid chromatographic assay method has been developed for the simultaneous quantitative determination of Tolperisone hydrochloride and Etoricoxib in tablets. **Methods:** The chromatographic separation of drugs was attained by using Eclipse plus C_{18} (150 mm × 4.6 mm, Agilent 5µm) column at room temperature. The composition of mobile phase was mixture of 0.035M triethylamine (pH 3.0 adjusted with ortho-phosphoric acid) and acetonitrile in ratio of 70:30 v/v and flow rate of mobile phase 1.0 ml/min with isocratic elution. The signal of eluents was observed at 290 nm by using diode array detector. **Results:** The retention time of Tolperisone hydrochloride and Etoricoxib were found to be 2.826 min and 7.566 min, respectively. The linearity ranges for both drugs were found to be 5-15 µg/ml and the percent recoveries were found to be 99.39% and 99.15% for Tolperisone hydrochloride and Etoricoxib respectively. Various forced degradation conditions, such as alkali hydrolysis, acid hydrolysis, thermal degradation, photolytic degradation, and oxidative degradation, were applied to the drug ingredients and drug formulation. The degradants were efficiently separated from the drugs by using optimized chromatographic conditions. The developed method was validated as per recommendation parameters of International council on harmonization guidelines Q2(R1). **Conclusion:** The validation parameters indicate that the drug substances were efficiently separated from its degradants and developed method can be routinely applied for the simultaneous quantitative determination of Tolperisone hydrochloride and Etoricoxib in combined tablet formulation in the quality control laboratory.

Key words: Quality control laboratory, Tolperisone hydrochloride, Etoricoxib, RP-HPLC-DAD method, Validation, ICH guidelines.

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INTRODUCTION

Etoricoxib is a selective inhibitor of Cyclooxygenase-2 (COX) belongs to the class of nonsteroidal anti-inflammatory drug,[1] chemically 5 – chloro-2-6(-methylpyridin-3-yl)-3-[4-(trideuteriomethylsulfonyl) phenyl]pyridine.[2] It helps to reduce pain and swelling in the joints and muscles. Tolperisone hydrochloride is a centrally acing muscle relaxant, chemically 2-methyl-1-(4-methylphenyl)-3-piperidin-1-ylpropan-1-one; hydrochloride it has been used in the treatment of spasticity and muscle spasm.[3] Etoricoxib is official in Indian pharmacopeia 2018.[4] Literature survey reveals that analytical method reported for the determination of Etoricoxib and Tolperisone hydrochloride as a single were, UV-spectrophotometric,[5] simultaneous equation method,[6] Q-analysis method,[7] "HPLC",[8] "RP-HPLC",[9] LC/MS-MS,[10] HPTLC[11] method. For the combination of Etoricoxib and Tolperisone hydrochloride no single approach has been described. The goal of this project is to develop and validate fast and robust stability indicating "RP-HPLC" method for simultaneous estimation of Tolperisone hydrochloride and Etoricoxib in pharmaceutical dosage form. The suggested analytical method on HPLC was used to estimate Tolperisone Hydrochloride and Etoricoxib in bulk and marketed formulation at the same time. ICH Q2 (R1)[12] guidelines were used to validate the developed method. Figure 1 depicts the chemical structure of Etoricoxib and Tolperisone hydrochloride.

MATERIALS AND METHODS

Chemicals and Reagents

Tolperisone Hydrochloride and Etoricoxib as reference standards procured as gift sample from Vaikunth Chemicals, Gujarat, India and Mylan Labs Ltd. Gujarat, India, respectively. Pharmaceutical dosage form of Tolperisone Hydrochloride and Etoricoxib (Label claim 150/60 mg) was procured from local drug store. HPLC Grade Methanol, Acetonitrile and water (Mili-Q) were purchased from Rankem Labs and Merck Life Science Pvt. Ltd, India. Sodium Hydroxide, Hydrogen Peroxide, Hydrochloric Acid used were AR grade and procured from the Central Drug House (P) Ltd. and Rankem Labs, respectively. The calibrated glassware was used in this study.

Instrumentation

The HPLC chromatograph (1260 Infinity, Agilent Technologies Ltd.) consisting a gradient pump, auto sampler, photo diode array detector, thermostatic control column oven. The drugs were separated on an Eclipse plus C_{18} (150 mm × 4.6 mm, Agilent 5µm) column and measured using Open Lab CDS software. An electronic balance (ME204/A04, Shimadzu), an ultra sonicator (LMUC 6), a digital pH meter (EQ-610, Lab line), a Centrifuge (CPR-24 Plus, Remi) and water purification system (Mili-Q) were used in this study.

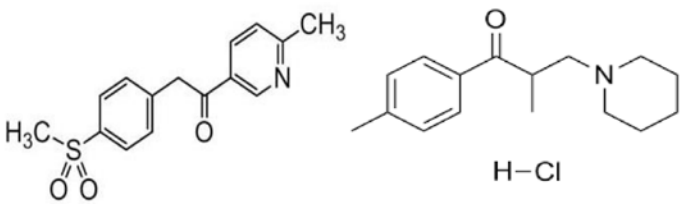

Figure 1:(A): Chemical structure of Etoricoxib and (b): Tolperisone hydrochloride.

Mobile Phase Preparation

In 1000 ml glass volumetric flask 5.0ml triethylamine was measured and inserted. The compound was dissolved in water and the pH 3.0 of buffer solution was adjusted with ortho-phosphoric acid. The buffer solution was filtered through 0.45 µm membrane filter and degassed before used. The mobile phase comprised of a mixture of 0.035M triethylamine (pH 3.0 adjusted with ortho-phosphoric acid) and acetonitrile in ratio of 70:30 v/v.

Preparation of Stock and Standard Solution

An equivalent amount of 75 mg of Tolperisone hydrochloride and of 30 mg Etoricoxib were weighed and transferred to 50 ml volumetric flasks separately. The drugs were sonicated to dissolve in 20 ml of methanol and diluted up to the mark with methanol to get 1500 µg/ml concentration and 600 µg/ml concentration of each drug respectively. The solutions were further diluted with diluent (0.035 M triethylamine pH 3.0 and acetonitrile in 60:40 v/v ratio) to obtain the final concentration of 150 µg/ml Tolperisone hydrochloride and 60 µg/ml of Etoricoxib respectively.

Chromatographic Conditions

The RP-HPLC method was carried out with isocratic mode using a mobile phase of 0.035M triethylamine, pH 3.0: acetonitrile (70:30 v/v) on Eclipse Plus C_{18} (150mm × 4.6mm, 5µm) column with 1 ml/min flow rate and eluents were monitored at 290 nm Figure 3 by using photodiode array detector. The volume of 10 µl sample injected using auto sampler.

Sample Preparation of Marketed Formulation

The marketed formulation (Etotol-150) tablets contained 150 mg of Tolperisone Hydrochloride and 60 mg of Etoricoxib. Weighed and finely powdered twenty tablets. Weighed accurately a quantity of tablet powder equivalent to 150mg of Tolperisone Hydrochloride and 60mg of Etoricoxib were transferred into 100 ml of volumetric flask, dissolved in 20 ml of methanol, sonicate with intermediate mixing for 15 min and diluted upto mark with methanol. Further centrifuge it at 3000rpm for 10 min. The solution was then diluted to concentration 150µg/ml and 60µg/ml respectively, using diluent.

METHODS

Preparation of Calibration Curve of Tolperisone Hydrochloride and Etoricoxib

Fixed standard aliquotes (2.5-7.5ml) of Tolperisone hydrochloride and Etoricoxib were transferred into five different glass volumetric flasks and diluted up to 50 ml with diluent to obtain concentration in range of 75-225µg/ml of Tolperisone hydrochloride and 30-90µg/ml of Etoricoxib. The fixed volume of sample was injected into the chromatograph with the help of auto sampler and chromatographic conditions. Calibration and regression equation were prepared by plotting peak areas versus concentrations and regression equations were calculated for Tolperisone hydrochloride and Etoricoxib.

Linearity and Range

The method's linearity was performed by analyzing five different concentrations 75-225µg/ml of the standard drug solutions of Tolperisone Hydrochloride and30- 90µg/ml of Etoricoxib separately. The area of peak versus concentration was used to plot the calibration curve. The correlation co-efficient, slope, intercept and regression line equations were found out from the calibration graph.

Accuracy

The accuracy parameter was performed by calculating percent recovery of Tolperisone Hydrochloride and Etoricoxib in pre-analysed sample. The known amount of pure drugs corresponding to 50, 100 and 150% of labelled value has been added into pre-analysed sample. The10 µl of sample solution was injected in chromatograph using auto sampler injector.

Precision

The repeatability of method was performed by analyzing Tolperisone hydrochloride (150 µg/ml) and Etoricoxib (60 µg/ml) respectively for six times (n=6) by developed RP-HPLC method and the %RSD was calculated. For the developed method precision was performed by intraday and inter day variation studies. Aliquotes of three different concentrations of Tolperisone hydrochloride (75 µg/ml, 120 µg/ml, 150µg/ml) and of Etoricoxib (30µg/ml, 50 µg/ml ,60µg/ml) were analysing responses on the similar day for three times and on three days in a row for intraday and inter day precision, respectively.

Quantitation Limits

The detection limit (LOD) and quantification limit (LOQ) were calculated as per equation given in the ICH guidelines: LOD=3.3(SD)/S and LOQ=10(SD)/S, where SD=standard deviation of peak area and S=average slope of calibration curve.

Robustness

Robustness of chromatographic method was checked by making small and deliberate changes in chromatographic conditions. Change in flow rate, temperature, wavelength and mobile phase pH were examined. In triplicate, injection of samples were made into chromatograph and chromatograms were developed using chromatographic conditions that were optimized

System Suitability Parameters

System suitability tests were performed to verify the chromatographic system's reproducibility. The 10µl sample solutions were repetitively injected into the chromatograph under optimal chromatographic conditions and parameters like retention time, area, theoretical plates, resolution and tailing factor were observed in order to evaluate the method's system suitability.

Stress Degradation

Stress degradation studies on tablet formulation were performed under various conditions like, hydrolytic, oxidative, photolytic and thermolytic stress conditions.

Acid Degradation

To perform acid degradation, 10 tablets were weighed and crushed. Equivalence of tablet crushed powder to one tablet was transferred to 100ml of volumetric flask. 5 ml of Mili-Q water was added and shaken gently. Then 1 ml 1N Hcl was added kept at room temperature for 24 hr. The solution was neutralized by adding 1N 1 ml NaOH. Then 50 ml methanol was added and sonicated for 15 min with intermittent

shaking. Then the volume was make up to the mark with Methanol. Further solution was centrifuged at 3000 rpm for 15 min. It was further pipette out, 5 ml of clear supernant to 50 ml volumetric flask and makeup to mark with diluent.

Alkali Degradation

To perform alkali degradation, 10 tablets were weighed and crushed. Tablet powder equivalent to one tablet was transferred in 100 ml of volumetric flask. After adding 5 ml of Mili-Q water shake it gently. Then 1 ml 1N NaOH was added kept at ambient temperature for 24 hr. The solution was neutralized by adding 1N 1 ml Hcl. Then 50 ml methanol was added and sonicated for 15 min with intermittent shaking. Then the volume was make up to the mark with Methanol. Further solution was centrifuged done at 3000 rpm for 15 min. It was further pipette out, 5 ml of clear supernant to 50 ml volumetric flask and makeup to mark with diluent.

Oxidative Degradation

To perform oxidative degradation, 10 tablets were weighed and crushed. Tablet powder equivalent to one tablet was transferred in 100 ml of volumetric flask. After adding 5 ml of Mili-Q water shake it gently. Then 1 ml 3 % hydrogen peroxide (H_2O_2) was added kept at 80°C for 30min. Then after about adding 50 ml methanol sonicate it for 15 min with periodic shaking. The volume was make up to final mark with solvent methanol. Further solution was centrifuged at 3000 rpm for 15 min. It was further pipette out, 5 ml of clear supernant to 50 ml volumetric flask and makeup to mark with diluent.

Thermal Degradation

To perform thermal degradation, 10 tablets were weighed and crushed. Tablet powder equivalent to one tablet was transferred to 100 ml volumetric flask. 5 ml of Mili-Q water was added and shaken gently. Then volumetric flask was kept at 80°C for 1hr. Then 50 ml methanol was added and sonicated for 15 min with intermittent shaking. Then the volume was make up to the mark with Methanol. The solution was centrifuged at 3000 rpm for 15 min. It was further pipette out, 5 ml of clear supernant to 50 ml volumetric flask and makeup to mark with diluent.

Photolytic Degradation

To perform photolytic degradation, 10 tablets were weighed and crushed. Tablet powder were exposed under ultraviolet light at 254 nm for 24 hr. After 24 hr. Tablet powder equivalent to one tablet was transferred to 100 ml volumetric flask. 5 ml of Mili-Q water was added and shaken gently. Then about 50 ml methanol was added and sonicated for 15 min with periodic shaking. Then the volume was make up to the final mark with solvent Methanol. The solution was centrifuged at 3000 rpm for 15 min. It was further pipette out, 5 ml of clear supernant to 50 ml volumetric flask and makeup to mark with diluent.

RESULTS

Development and Optimization of Analytical Method

The chromatographic separation and quantitation of drugs in existence of its degradants was done using isocratic mode on Eclipse plus C_{18} (150mm×4.6mm, Agilent 5µm) reverse phase column with 1.0 ml/min flow rate. The mobile phase having composition of 0.035M triethylamine (PH 3.0 adjusted with ortho-phosphoric acid) and acetonitrile (70:30v/v) shown efficient resolution peak in the existence of degradation products and impurities. The volume of 10µL sample solution was injected using auto sampler. The drugs were monitored at 290nm by using photodiode array detector. The time of retension of Tolperisone Hydrochloride and etoricoxib under optimized conditions was 2.826 min and 7.566 min, respectively. There was a straight baseline in the blank chromatogram of mobile phase and both the drug were effectively resolved in bulk and formulation (Figure 2). The parameters like, retention time, area, theoretical plates, resolution and tailing factor were observed for system suitability and found within limits stipulated limits according to International Council for Harmonisation (ICH) guidelines the parameters of system suitability were observed within the specified limits. (Table 1).

Linearity and Range

Linearity of analytical method was checked by analyzing five different concentrations levels of the standard drug solutions of Tolperisone hydrochloride and Etoricoxib separately. The linearity for Tolperisone hydrochloride was observed to be 75 - 225µg/ml and Etoricoxib was observed to be 30-90µg/ml. The correlation co-efficient and equations of regression were determined from the calibration curve. Figure 4 shows the linearity overlay.

VALIDATION

Precision and Accuracy

The values of %Relative standard deviation for of repeatability of method, intraday precision and interday precision were observed below 2% divulge that developed method is precise. The recovery experiments were carried out in order to determine accuracy of analytical method; known concentration of standard drug was added into pre-analysed sample at three separate levels,50, 100 and 150%. The percent recovery of drug was calculated from the regression equations (Table 2).

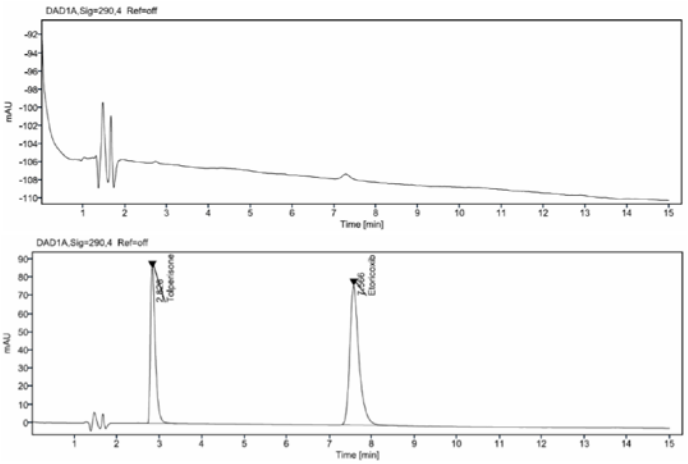

Figure 2: Typical HPLC chromatogram of blank and standard mixture of drugs (Retention time of Tolperisone Hydrochloride= 2.82 min. and Etoricoxib = 7.56 min)

Table 1: System suitability parameters of Tolperisone hydrochloride and Etoricoxib.

Parameters	Results of Tolperisone Hydrochloride	Results of Etoricoxib
%RSD of peak area	666.13	1153.81
%RSD of retention time	2.833	7.613
Theoretical plates (N)	3450	6455
Tailing factor (T)	1.71	1.43
Resolution (R)	---	16.72

Table 2: Accuracy data of Tolperisone Hydrochloride and Etoricoxib.

Parameter	Amount added	Amount recovered (µg/ml)	%Recovery Mean ± SD (*n*=3)	Mean % Recovery
Tolperisone hydrochloride				
150%	225.8ppm	224.2	99.29±0.56	
200%	300.4ppm	298.4	99.33±0.43	99.39±0.59
250%	375.5ppm	373.9	99.57±0.79	
Etoricoxib				
150%	90.2ppm	89.1	98.78±0.68	
200%	120.7ppm	118.9	98.50±0.76	99.15±0.65
250%	150.4ppm	150.7	100.19±0.52	

Sensitivity and Specificity

The standard deviation and the slope of the calibration plots were used to determine the detection and quantitation limits. The estimation of detection and quantitation limits (LOD) and (LOQ) were considered the ratio of signal –to-noise which were 3:1 and 10:1, respectively. LOD of Tolperisone Hydrochloride and Etoricoxib were 0.406 µg/ml and 0.167 µg/ml respectively. LOQ of Tolperisone Hydrochloride and Etoricoxib were 1.232 µg/ml and 0.5067 µg/ml respectively. The chromatograms of degradation test and assay results finding of tablets revealed that no interference was found with degradants and additives of tablet formulation. The peak purities and chromatograms of the stress sample indicate the specificity of analytical method. The robustness parameters of method were checked by injecting sample with small changes in the chromatographic conditions like. Change in temperature, wavelength, flow rate and mobile phase pH and the %Relative Standard deviation of each variable was calculated (Table 3).

Stress Degradation

The stress degradation studies indicate that the drugs were sensitive to, alkali, acid, oxidation, and thermolytic degradation conditions. The chromatograms of stress degraded drug samples shows good separation of both the drugs from its degradants. The chromatograms of different forced degradation conditions of sample shown that the drug substance were well separated from impurities and degradants (Figure 5). The % degradation of drugs were calculated by computing areas of drugs in each condition with the respective peak areas of drugs under normal condition.

Assay of Commercially Available Formulation

The sample was prepared from marketed formulation (tablets) prepared as per procedure mentioned earlier (Tolperisone Hydrochloride 150µg/ml and Etoricoxib 60 µg/ml). Assay of marketed tablets was performed in triplicate under the chromatographic conditions that are optimized and from the regression equation of calibration curves of Tolperisone Hydrochloride and Etoricoxib respectively. The average % content of Etoricoxib and Tolperisone Hydrochloride from curves of calibration were observed to be 99.80% and 99.08% respectively. None of the tablet excipient interfere with the drug molecule's analyte peak. The %Relative standard deviation for all method parameters were found to be within acceptable limits, which shows the validity of method and % content of drugs by this method are in acceptable limits.

Solution Stability

The stability of sample were evaluated at periodic intervals of 6hr,12hr and 24hr at ambient temperature. The sample's peak area was calculated

Table 3: Summary of method validation parameters.

Parameters	Tolperisone Hydrochloride	Etoricoxib
Linearity (µg/ml)	75-225	30-90
Slope (m)	4.5272	19.982
Intercept (c)	-0.544	-8.0571
Correlation coefficient (r^2)	0.9995	0.9995
Regression equation	Y=4.5272x-0.544	Y=19.982x-8.0571
LOD (µg/ml)	0.406	0.167
LOQ (µg/ml)	1.2323	0.5067
Repeatability (%RSD, *n*=6)	0.2547	0.3440
Precision (%RSD)		
Intraday (*n*=3)	0.198-0.548	0.260-0.789
Interday (*n*=3)	0.380-0.860	0.458-1.0
Robustness		
Flow rate change	0.36-0.57	0.31-0.81
Temperature change	0.11-0.47	0.17-0.30
Wavelength change	0.5-0.78	0.2-1.2
Mobile phase pH	0.9-1.5	0.5-1.25

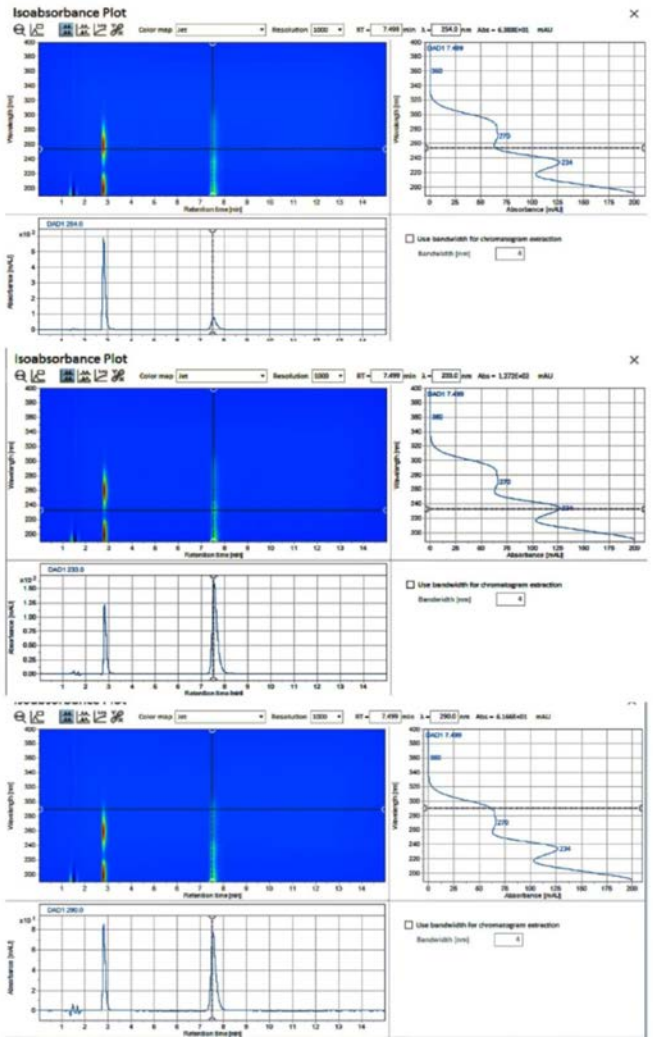

Figure 3: Isoabsorptive wavelength selected as 290 nm for Etoricoxib and Tolperisone hydrochloride.

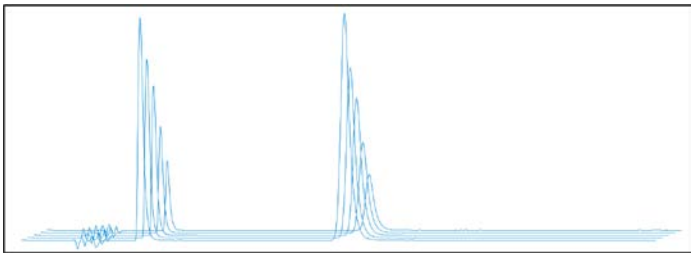

Figure 4: Linearity overlay.

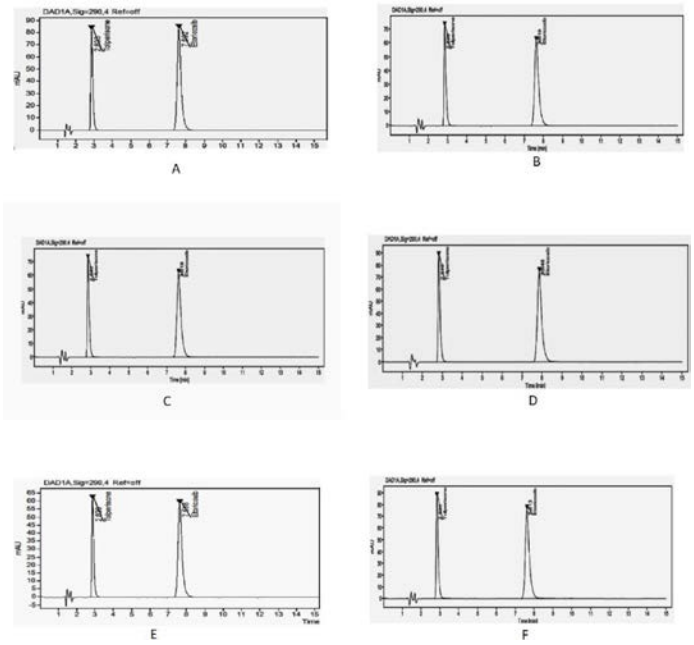

Figure 5: Chromatograms of acid degradation (A); alkali degradation (B); Photolytic degradation in UV expose (C) and UV control (D); oxidative degradation (E) and thermal degradation (F), where retention time of Tolperisone Hydrochloride= 2.82 min. and Etoricoxib = 7.56 min

at particular intervals by using chromatographic conditions that are optimized. Finding of the results says that mobile phase and sample solution were stable even after 24hr at an ambient temperature.

DISCUSSION

In the present developed method, the selected drug substances having different solubility, dissociation constants and polarity of drug molecules ultimately different physiochemical properties. The physiochemical properties of each one of drug molecules were investigated and appropriate analytical wavelength was identified using detector called photodiode array for the simultaneous quantitative estimation of Etoricoxib and Tolperisone Hydrochloride. Various compositions of mobile phases were explored for successful separation of drugs in the existence of degradants based on the available literature of physiochemical properties. The effect of chromatographic factors like wavelength detection, stationary phase, flow rate, pH of mobile phase and composition of mobile phase were evaluated so that chromatographic conditions get optimized. In this study, firstly various compositions of mobile phase having polar solvents like Acetonitrile, water and methanol in different ratios and different pH were tried for the simultaneous elution of selected drug substances. When mixture of water and methanol was used as

Table 4: Summary of forced degradation study.

Description of stress conditions	Content of drug after degradation		Peak purity index	
	Tolperisone HCL	Etoricoxib	Tolperisone HCL	Etoricoxib
Acidic/ 1N HCL/80°C/30 min	73	86	0.9968	0.9956
Alkali /1N NaOH/80°C/30 min	81	81.2	0.9989	0.9934
Oxidative/3%H_2O_2 /80°C/30 min	66	77.39	0.9993	0.9925
Thermal/1hr/80°C	54	55.21	0.9934	0.9943
UV Exposure /24hr	97	98	0.9931	0.9912
UV Control / 24hr	98	97	0.9910	0.9947

mobile phase peaks were merged and shows broadening. In mixture of Acetonitrile: water peaks shown poor resolution. So, buffer solution was tried so that chromatographic conditions got optimized. The mobile phase with composition of 0.035M triethylamine (pH-3.0 adjusted with O-phosphoric acid) and Acetonitrile (70:30v/v) shown efficient and fast separation of selected drug substances and their degradants formed during forced degradation study.

Method's system suitability was examined by evaluating the parameters such as, retention time, area, theoretical plates, resolution and tailing factor. The current project was performed on Eclipse C_{18} (150mm × 4.6mm, Agilent 5µm) column at ambient temperature which showed better reproducibility and low back pressure. The outcome of system suitability parameters were found to be within acceptable specified limits as per recommendations of International Council on Harmonization (ICH) guideline. The straight baseline was found in the blank chromatogram of mobile phase and both drugs were effectively resolved in bulk and formulations.

The estimated detection and quantification limit values confirmed that method was sufficiently sensitive for the detection of drug in the existence of their degradants. Also, percent recoveries of the drugs were observed to be within acceptable limits. Method's Specificity was assessed by injecting blank, standard, sample and performing forced degradation. The statistical data obtained during robustness study indicate that the method which is developed is robust.

In the degradation study, it was found that the both drugs were susceptible to hydrolysis, oxidation and thermolysis.

CONCLUSION

A specific, rapid, precise and accurate stability-indicating liquid chromatographic method was developed for the quantitation of Tolperisone hydrochloride and Etoricoxib in the combined tablets detector called diode array. The ICH recommendations parameters of method validation suggests that successful separation two drugs with its degradants was possible using the proposed method. The developed stability-indicating RP-HPLC method could be frequently used in the department of quality control for the quantitative determination of drugs.

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ABBREVIATIONS

ICH: International Conference on Harmonization; **RP-HPLC:** Reversed Phase High Performance Liquid Chromatography; **DAD:** Diode Array Detector; **LOD:** Limit of Detection; **LOQ:** Limit of Quantitation; **SD:** Standard Deviation; **RSD:** Relative Standard Deviation; **RS:** Reference Standard; **HPTLC:** High Performance Thin Layer Chromatography; **LC-MS:** Liquid Chromatography Mass Spectrometry; **UV:** Ultra-Violet; **AR:** Analytical Reagent; **RS:** Reference standard.

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