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Validated Inherent Stability Indicating Reversed Phase HPLC-DAD Method for Simultaneous Determination of Tolperisone Hydrochloride And Diclofenac Sodium in Marketed Formulation

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ABSTRACT

The new method for simultaneous establishment of tolperisone hydrochloride (TPS) and diclofenac sodium (DLF) has been evolved by reverse phase HPLC from combination drug product. The separation achieved on C18 (4.6 x 250mm, 5µm particle size) column with isocratic elution of mobile phase comprising methanol:water, in the ratio of 80:20 v/v (pH 3.0 adjusted with OPA). The mobile phase flow rate was maintained at 1.0 ml/min and the analyte concentration was measured at detection wavelength 273 nm. The calibration curves were linear over the concentration range of 30–150 µg/ml and 10–50 µg/ml for TPS (n = 3) and DLF (n = 3), respectively.The TPS and DLF were exposed to different stress condition like thermal, photolytic, hydrolytic, and oxidative stress conditions and samples were analyzed by proposed method.The stressed sample demonstrated the specificity of assay method in presence of degradant products with no interferences were observed from its stress degradation products.The detection limits were found to be0.99 µg/ml and 0.41 µg/ml and quantification limits were found to be 3.42 µg/ml and 1.53 µg/ml.Analytical performance of the proposed HPLC procedure was thoroughly validated in terms of linearity, precision, accuracy, specificity, robustness, detection and quantification limits. The proposed method was suitable for quantitative determination and stability study ofTPS and DLF in commercial tablets.

Key words: *Tolperisone hydrochloride; Diclofenac sodium; Stability indicating assay; Forced degradation; HPLC; Diode array detection*

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INTRODUCTION

Stability testing forms an important part of drug product process development. The purpose of stability testing isto provide evidence on how the quality of a drug substance or drug product varies with time under the influence of a variety of environmental factors such as temperature, humidity and light, enables recommendation of storage conditions, retest periods, and shelf lives to be established. The two mainaspects of drug product that play an important role in shelflife determination are assay of active drug, and degradants generated, during the stability study. The assay of drug productin stability test sample needs to be determined using stability indicating method.[1]In recent times, there is an increasing tendency towards the development of stability-indicating assays, using the approach of stress testing as incorporated in the International Conference on Harmonization (ICH) guideline. Even this approach is being extended to drug combinations, to allow accurate and precise quantitation of multiple drugs, their degradation, and interaction of products. Tolperisone hydrochloride (TPS) is chemically designated as(R, S) 2-methyl-l-(4 methyl phenyl)-3- (l-piperidyl)propan -l-one(Fig.1). TPS,an antispasmodic agent and a central muscle relaxant suitable for cerebral arteriosclerosis and for treating extrapiramidal movement disorders.

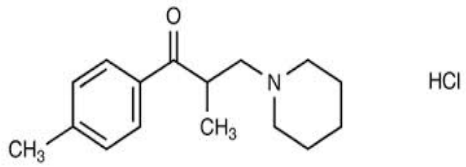


Fig. 1. Chemical Structure of Tolperisone hydrochloride (TPS)

Tolperisone has the unique property of mediating muscle relaxation without concomitant sedation and it does not cause in co-ordination, weakness and mental confusion or withdrawal phenomena, in contrast to other muscle relaxants.It is official in Japanese pharmacopoeia. It is estimated by the potentiometric method as per JP. Diclofenac sodium (DLF) chemically it is 2-[2, 6 dichlorophenylamino] benzene acetic acid sodium salt (Fig. 2). It is classified as anon-steroidal anti-inflammatory agent. It is used to treat pain or inflammation caused by arthritis or ankylosingsypondylitis. DLF is official in IP, BP and USP.IP and BP describes the liquid chromatography method and USP describe the potentiometric method for estimation of DLF. The combination capsule formulation containing TPS and DLF has potential antispasmodic and anti-inflammatory activity. It is extensively used for the treatment of patients with acute musculoskeletal spasm associated with lower back pain.

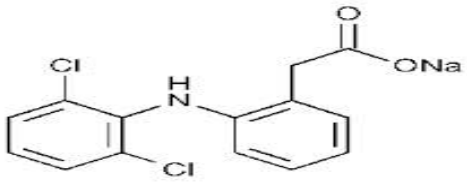


Fig. 2. Chemical Structure of Diclofenac sodium (DLF)

There are several UV,HPLC and HPTLC method reported for analysis of TPS individually or in combination with other drugs.[2-7] Analytical method existing for diclofenac sodium included determination by UV, HPLC or in combination with other drugs.[8-11]To the foremost our learning no article related to the HPLC stability-indicating method for the simultaneous determination combinations of these two drugs has ever been mentioned in the literature, and not official in any pharmacopoeia; hence no official method available for TPS and DLF in their combined dosage forms.[12-17]
A developed HPLC method with high sensitivity and selectivity will be useful for the estimation of TPS and DLF in combined pharmaceutical dosage form in presence of their degradation product. Therefore the aim of study was to develop and validate sensitive, precise, accurate and specific HPLC-DAD method for determination of TPS and DLF in combined dose pharmaceutical formulation.

MATEIAL AND METHODS

Chemicals and reagents

Working standards of pharmaceutical grade TPS, was received as a gift sample from Richard themis pharmaceuticals Pvt.Ltd,Vapi Gujarat, India and DLF was obtained as a generous gift from EmcurePharmaceuticals Ltd, Pune.A combination product containing the two drugs was purchased from local pharmacy shop. Sodium hydroxide, hydrochloric acid and hydrogen peroxide were purchased from Qualigens Fine Chemicals (Glaxo Ltd.).HPLC grade acetonitrile, water and methanol were purchased from Merck (Darmstadt, Germany).

Equipment and chromatographic condition

The modular HPLC system used was equipped with Waters 510 HPLC pump (Waters Chromatography Division, Milford, MA, USA), a Rheodyne injector (20 µl), solvent degasser and PDA 6000 LP detector. A DataAce Chromatography data system was used to record and evaluate the data collected during and following chromatographic analysis. The chromatographic separation was achieved on a Kromasil C-18, (250 mm × 4.6 mm i. d., and 5µm particle size) column using a mixture of methanol: water in the ratio 80:20 v/v (pH adjusted to 3.0 with OPA) as mobile phase at a flow rate of 1.0 ml/min. The eluent was monitored using photodiode array (PDA) detection at a wavelength of 273 nm. The mobile phase was filtered through a 0.45 µm nylon filter prior to use and sonicated using an ultrasonic bath (Biomedica, India). A precision water bath equipped with MV controller (i-therm, Biomedica, India) was used to carry out selected reactions in solution during stress degradation study. Thermal stability study was carried out in dry air oven (Biotechnics BTI–20D, Mumbai, India). Other equipments used were sonicator (Biomedica, India), analytical balance (Schimadzu AUX 220, Japan) and auto pipettes (Eppendorf, Hamburg, Germany).

Preparation of standard and stock solution

A stock solution of TPS and DLF (750 µg/ml) and (1000 µg/ml) was prepared by accurately weighing approximately 37.5 and 25 mg of TPS and DLF into a 50 ml A-grade volumetric flask and making up the volume with HPLC grade mobile phase. The stock solution was protected from light using aluminium foil and stored for one week at 4°C and was found to be stable during this period. Aliquots of the standard stock

solution of marketed formulation were transferred using A-grade bulb pipette into 25 ml volumetric flasks and the solutions were made up to volume with mobile phase to give final concentrations of 30,60,90,120 and 150 µg/ml for EPS and 10,20,30,40 and 50 µg/ml for DLF.

Forced degradation studies of API and capsule contents

In order to determine whether the analytical method and assay were stability-indicating, marketed capsule formulation, TPS and DLF active pharmaceutical ingredient (API) powder were stressed under various conditions to conduct forced degradation studies. In all cases, API (1mg/ml) and tablet powder were accurately weighed, subjected to forced degradation and prepared for analysis. The same stress conditions were applied to placebo and blank solution. Methanol was used as co-solvent in all studies.All stressed solutions of API and marketed formulation were withdrawn periodically during stress study and subjected to analysis after suitable dilution of stressed samples to yield final concentrations of 60 and 20 µg/ml for TPS and DLF, respectively and filtered before injection in the chromatographic system.

Acid degradation studies

Solutions for acid degradation studies were prepared in methanol and 0.5M hydrochloric acid (20:80, v/v) and the resultant solutions refluxed for 2h at 70°C.

Alkali degradation studies

Solutions for alkali degradation studies were prepared in methanol and 1M sodium hydroxide (20:80, v/v) and the resultant solutions refluxed for 1h at 70°C.

Neutral degradation studies

Solutions for neutral degradation studies were prepared in methanol and water (20:80, v/v) and the resultant solutions refluxed for 3 hat 70°C.

Oxidation

Solutions for oxidation studies were prepared in methanol and 6% H_2O_2 (20:80, v/v) and the resultant solutions were kept for 5h at room temperature.

Photolytic degradation

API and tablet powder in solid form were exposed to shorter and longer UV radiation to determine the effect of light irradiation on the stability of TPS and DLF. Approximately 50 mg of API powder was spread on a glass dish in a layer that was less than 2mmthick.Capsules were prepared in the same way. All samples for photostability testing were placed in a light cabinet and exposed to light for 24h. Control sample protected with aluminium foil was also placed in the light cabinet and exposed concurrently. Following removal from the light cabinet, all samples were prepared for analysis as previously described.

Temperature stress studies

Capsules and API powder were exposed to dry heat in an oven at 70°C for 6 h. The capsules and API powder were removed from the oven and the contents of 20 capsules were removed and mixed. An equivalent weight of capsule powder and API powder were then prepared for analysis as previously described.

Development of method

HPLC studies were carried out on all the reaction solutions containing API individually, and on a marketed formulation. The separations were achieved by isocratic elution using methanol: water (80:20 v/v, pH adjusted to 3.0 with OPA) as a mobile phase. It was filtered through 0.45 µm nylon filter and degassed before use. The injection volume was 20µl and mobile phase flow rate was 1.0 ml/min. The detection was carried out at 273 nm.

Preparation of tablets for assay

Twenty tablets were weighed, crushed and mixed in a mortar and pestle for 20 min. A portion of powder equivalent to 37.5mg TPS and 12.5mg DLF was accurately weighed and transferred into each of six25 ml A-grade volumetric flasks, 15 ml mobile phase was added and sonicated for 20 min for complete dissolution of the TPS and DLF and the solutions were then diluted up to volume with mobile phase. Aliquots of the solution were filtered through a 0.45µm nylon filter. From the filtrate, 1ml of the filtered solution was transferred to a 25ml A-grade volumetric flask and made-up to volume with mobile phase, to yield final concentrations of drugs in the range of linearity.

Validation of the method

The method was validated for linearity, precision (inter-day, intra-day), accuracy, specificity, selectivity, LOD, LOQ and robustness. Standard plots were constructed for both TPS and DLF in the range of 30-150

and 10-50 µg/ml, respectively. The system precision was evaluated by six replicate injections of the standard solution. The method precision was studied by injecting six standard solutions of same concentration. The results for method precision and system precision were expressed in terms of percent relative standard deviation. The intraday precision was evaluated by analyzing the capsule sample at three different time interval on the same day. The inter-day precision of the method was studied by analyzing the capsule samples in three consecutive days. Accuracy was determined by fortifying the marketed formulations with three known concentrations of the pure drugs. Further, the specificity of the method was assessed by study of the resolution factor of the drug peaks from nearest resolving peaks. The selectivity was determined by checking peak purity of all the peaks, including those of degradation products, using a PDA detector.

RESULTS AND DISCUSSION

The immediate interest in developing present stability indicating HPLC method was to accomplish resolution between TPS and DLF and its degradation product. The preferred chromatographic condition chosen was established on symmetry of peak shape and contraction of chromatographic analysis. The mobile phase consisting mixture of methanol: water in the ratio 80:20 v/v (pH adjusted to 3.0 with OPA) as mobile phase at a flow rate of 1.0 ml/min.The selection of organic solvent based on its affinity towards supportive UV transmittance, low viscosity and provides acceptable resolution between two drugs.The recommended chromatographic conditions applied an available C18 column with least use of organic solvent. The established method demonstrates no interferences from any possible forced degradation products. The results obtained from the study it can be concluded that our proposed method was closer to the ideal conditions of an assaying method for routine industrial use.

Development and optimization of stability-indicating HPLC method with degradation behavior

Anisocratic method was found necessary to optimize the separation of major degradation products formed under various stress conditions. The best resolution was achieved with initial run of methanol: water in the ratio of (80:20 v/v, pH 3.0 adjusted with OPA). The obtained chromatogram is represented in fig. 3 shows the Rt of TPS and DLF at 3.21 and 9.10 respectively.

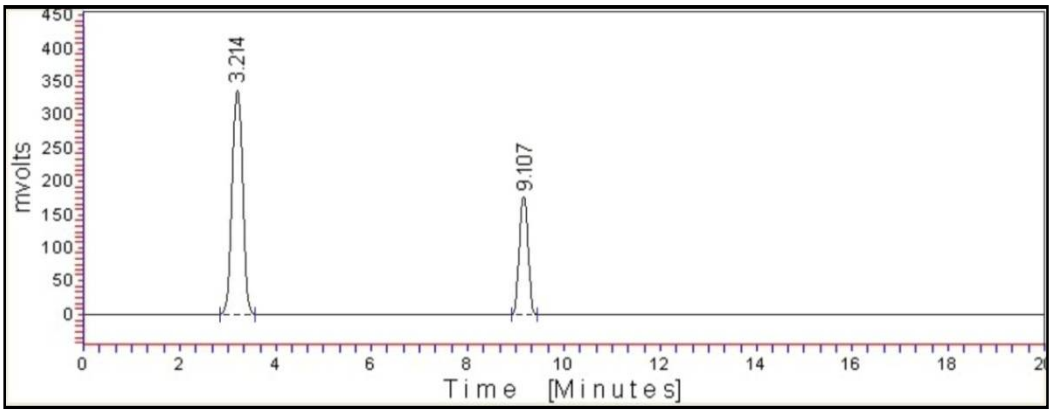
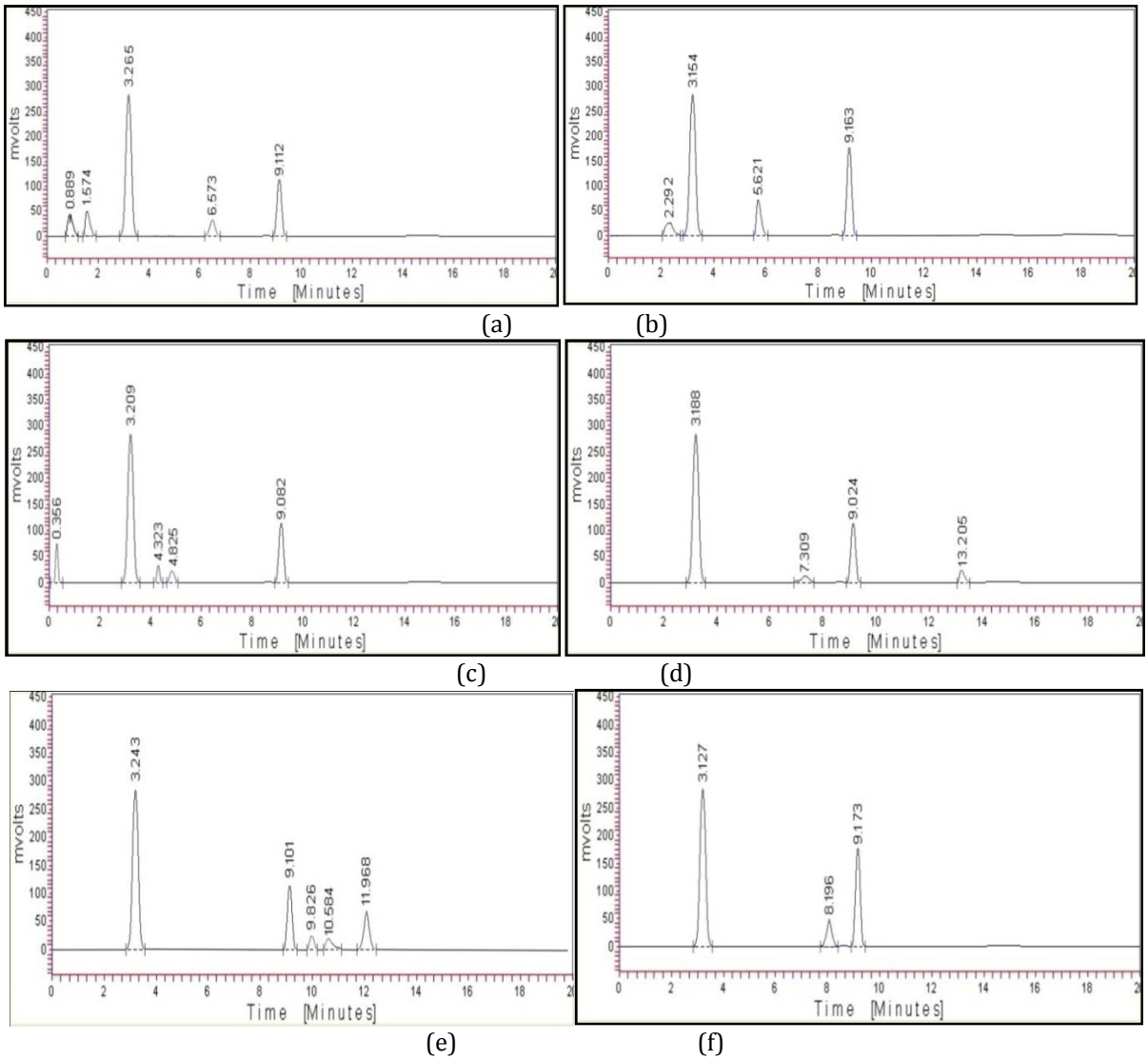


Fig. 3. Typical Chromatogram of EPS (Rt = 3.21 min) and DLF (Rt= 9.10 min) respectively.

The method worked well with the mixture of degradation solutions and was even applicable to degraded formulations. Fig. 4(a–f) shows the obtained chromatographic resolution of TPS and DLF from its degradation product generated during various stress conditions. HPLC studies on API (TPS and DLF) and tablets under different stress conditions indicated the following degradation behavior. Both the drugs were found to be highly labile and showed degradation within 2h at 70oC in 0.5M HCl. TPS showed little higher degradation as compared to DLF. The major degradation products formed were at retention times (RTs) 0.8, 1.5 and 6.5 min. The API and tablet formulation was refluxed in alkaline condition. TPS showed degradation within 3.5hat 70^{o}Cin 1M NaOH, while DLF was found stable in alkaline condition. The major products appeared at RTs 2.2, and 5.6 min.



(a) (b) (c) (d) (e) (f)

Fig. 4.Indicates the obtained chromatograms of stress studies of TPS and DLF as: a) Acid degradation b) Alkali degradationc) Neutral degradation d) Oxidative degradation e) Photolytic degradation f) Thermal degradation

Sufficient amount of degradation was observed upon refluxing the combination for 3h at 70°C. DLF showed little higher degradation as compared to TPS. The major degradation products appeared at RTs 0.3, 4.3 and4.8 min.The drugs showed sufficient amount of degradation in 6% H_2O_2 for 5h at room temperature. DLF found to be more prone to oxidation than TPS. The two major degradation products appeared at 7.3 and 13.2 min.API and tablet formulation were exposed to UV radiation for 24 h, similar to oxidative degradation, DLF was found to be more susceptible to photolytic degradation than TPS. The major degradation products found at 9.8, 10.5 and 11.9 min. Enough degradation was observed when the combination was exposed to dry heat at 70°C for 6h. DLF showed appreciable stability and did not influence to thermal degradation while TPS undergone degradation. The major degradation products observed at 8.1 min.

Validation of the developed stability-indicating method

The analytical method was validated with respect to parameters such as linearity, limit of quantitation (LOQ), limit of detection (LOD), precision, accuracy, selectivity, recovery and robustness/ruggedness.

Linearity

Linearity was established by least squares linear regression analysis of the calibration curve The five point constructed calibration curves were linear over the concentration range of 30–150µg/ml and 10–50µg/ml for TPS (n = 3) and DLF (n = 3), respectively. Peak areas of TPS and DLF were plotted versus their respective concentrations and linear regression analysis was performed on the resultant curves. Correlation coefficients (n=3) were found to be 0.999 for both the drugs with %RSD was found to be < 2.

Typically, the regression equations were: $y = 2027x + 376.4$ ($r^2 = 0.999$) for TPS and $y = 710.5x - 0.190$ ($r^2 = 0.999$) for DLF, respectively.

LOD and LOQ

The detection and quantification limits were evaluated from calibration curves plotted in concentration ranges of 30–150µg/ml for TPS (n = 3) and 10–50µg/ml for DLF(n = 3). The approach based on the standard deviation of the response and the slope was used for determining the detection and quantitation limits and the formulae used were LOD= 3.3σ/S and LOQ= 10σ/S (where σ = standard deviation of response and S = slope of calibration curve). The LOD for TPS and DLF were found to be 0.99µg/ml and 0.41µg/ml, respectively. The LOQ that produced the requisite precision and accuracy was found to be 3.42µg/ml and 1.53µg/ml for TPS and DLF, respectively.

Precision

The intra- and inter-day variability or precision data were summarized in table 1 and 2, respectively and were assessed by preparation of standard solutions to produce solutions of three different concentrations of TPS and DLF. Repeatability or intra-day precision was investigated by injecting six replicate samples of eachof the samples of three different concentrations. Inter-day precision were assessed by injecting the sample of three different concentrations over three consecutive days.

Table.No.1: Intra-day Precision Data

Parameters	TPS			DLF		
	Concentration (µg/ml)			**Concentration (µg/ml)**		
	48	**60**	**72**	**16**	**20**	**24**
% Estimated	99.55	99.08	99.32	99.42	99.20	99.53
S. D.	± 0.5783	± 0.6610	± 0.5880	± 0.5593	± 0.6199	± 0.5040
C. V	0.5809	0.6671	0.5920	0.5625	0.6249	0.5063

* Mean of six determinations, S.D: Standard Deviation, C.V: Coefficient of variance

Table.No.2: Inter-Day Precision Data

Parameters	EPS			DLF		
	Concentration (µg/ml)			**Concentration (µg/ml)**		
	48	**60**	**72**	**16**	**20**	**24**
% Estimated	99.53	99.58	99.19	99.32	99.52	99.38
S. D.	± 0.5006	± 0.5523	± 0.3003	± 0.9953	± 0.7219	± 0.6342
C. V	0.5030	0.5546	0.3028	1.0021	0.7253	0.6381

* Mean of six determinations, S.D: Standard Deviation, C.V: Coefficient of variance

3.3.4. Accuracy

Accuracy study was performed by standard addition method by adding pure drug in a powder of marketed formulation at three different levels 80%, 100% and 120%. In each case, the percent relevant error and %RSD was calculated and found to be less than 0.56for TPS and 0.82 for DLF. The data obtained from recovery study for the determination of each compounds of interest are summarized in table 3.

Table No.3: Statistical Validation for Recovery Study

Level of recovery	% Mean Recovery		Standard Deviation		% R.S.D.		S.E	
	TPS	**DLF**	**TPS**	**DLF**	**TPS**	**DLF**	**TPS**	**DLF**
80 %	99.00	99.16	± 0.551	± 0.815	0.556	0.821	0.318	0.470
100 %	99.27	99.84	± 0.431	± 0.056	0.434	0.056	0.249	0.032
120 %	98.92	99.68	± 0.141	± 0.250	0.142	0.250	0.081	0.144

*Average of three determinations, R.S.D: Relative Standard deviation, S.E.M: Standard error of mean

Specificity

The results of stress testing studies in addition to that of monitoring standard solutions of each drug in the presence of their degradants indicated a high degree of specificity of this method for both TPS and DLF. The degradation product(s) of each of the parent compounds was found to be similar for both the tablets and API powders assessed. The method has sufficient specificity and selectivity as the two drugs and even degradation products were well separated from each other, with the resolution factor of >2 in all cases. All the peaks were pure, which was proved through PDA purity studies. Data of peak purity index and purity threshold values indicates the degradants peaks are well separated from the drug peak.

The established mass balance study ensured that all degradants were adequately detected. The above study is shown in table 4.

Table No. 4: Mass balance and Peak Purity study

Stress Condition	% degradation		Purity Angle		Purity Threshold		% Assay		Mass Balance	
	TPS	**DLF**	**TPS**	**DLF**	**TPS**	**DLF**	**TPS**	**DLF**	**TPS**	**DLF**
Acid 0.5M HCl for 2h at 70°C.	23.00	16.19	0.099	0.364	0.812	0.936	76.03	82.3	99.03	98.49
Alkaline 1M NaOH for 1h at 70°C	22.73	0.0	0.285	0.142	0.952	0.823	76.19	99.55	98.92	99.55
Neutral H_2O for 3 h at 70°C	16.27	21.9	0.163	0.574	0.679	0.101	82.48	76.91	98.75	98.81
Oxidative 6% H2O2 for 5h at 70°C	5.96	7.82	0.428	0.243	1.163	0.982	93.41	91.17	99.37	98.99
Photolytic 24h in UV- radiation	4.98	25.11	0.255	0.311	0.922	0.874	94.78	74.06	99.76	99.17
Thermal 6h at 70°C	15.31	0.0	0.326	0.426	0.469	1.081	83.19	99.13	98.5	99.13

*Average of three determinations

3.3.6. Robustness

The robustness of the method was investigated under a variety of conditions including changes of detection wavelength, flow rate and of organic phase composition. The degree of reproducibility of the results obtained as a result of small deliberate variations in the method parameters and by changing analytical operators has proven that the method is robust as R.S.D was found to be <1%.

CONCLUSION

In this work proposed and efficient HPLC DAD method was established validated for its simultaneous assessment of TPS and DLF. It was observed from the literature survey that no official method was reported for present combination. Hence sincere efforts were plan to validate quantitative determination of TPS and DLF. In summary method was validated and gratifying results were obtained for all components tested. The retrieve data declared current approach was linear response in fixed range with accurate and precise. The establish method reports least possible use of organic solvents thus contribute superlative security and environmental concerns. The results indicated the suitability of method to study stability of TPS and DLF under various forced degradation conditions. The result of stress testing undertaken according to the International Conference on Harmonization (ICH) guidelines reveals that the developed method was selective and stability-indicating. The proposed method has the ability to separate these drugs from their degradation products. The method could be applied with success even to the analysis of marketed products, as no interference was observed due to excipients or other components present. It can be accomplished that the recommended methods has extreme assurance as rapid analytical tools for simultaneous estimation of TPS and DLF in their combined pharmaceutical formulations, principally for quality control laboratories.

CONFLICT OF INTEREST

We have no conflict of interest to declare.

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