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Research Article

Bioanalytical Method Development and Validation for Simultaneous Estimation of Tolperisone Hydrochloride and Diclofenac Sodium by RP – HPLC in Combined Pharmaceutical Dosage Form

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ABSTRACT

A simple, accurate and cost effective bioanalytical isocratic reverse phase high performance liquid chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of diclofenac sodium (DFS) and tolperisone hydrochloride (TOL) in tablet dosage forms.The method developed in Shimadzu HPLC system on C-18 column (4.6X250mm, 5µ,100A°) with a mobile phase composed of methanol:0.1% formic acid in water (90:10 V/V) and flow rate of 1.0mL/min and injection volume 20µL.Detection of common wavelength was carried out at isobestic point of both the drugs at 255nm. The retention time of diclofenac sodium and tolperisone hydrochloride were found to be 4.2min and 2.6 min respectively. Plasma spiking studies were also performed using rat plasma. This developed method was validated for LOD, LOQ,linearity,precision,reproducibility,specificity, systemsuitability, robustness and ruggedness as per the ICH guidelines.

Keywords: RP- HPLC; Diclofenac sodium (DFS); Tolperisone hydrochloride (TOL); Rat plasma ; Validation

1. INTRODUCTION

Diclofenac sodium is a non-steroidal anti-inflammatory drug (NSAID) which is advocated for use in painful and inflammatory rheumatic conditions. It is a derivative of phenyl acetic acid. It appears in various dosage forms such as orally, rectally and parenteral. Chemically it is known as 2-{2-(2,6-dichlorophenyl)amino phenyl}acetic acid [1]. Tolperisone is a piperidine derivative. It is a centrally acting muscle relaxant agent which is widely used in spasmolytic. Chemically it is known as 2-methyl-1-(4 methylphenyl)-3-piperidin-1-yl-propan-1-one.[2] Based on the extensive literature survey, HPLC methods are available for analyzing the diclofenac sodium and tolperisone with a mobile phase of acetonitrile : methanol: phosphate buffer in pharmaceutical dosage forms. Hence it is felt necessary to develop a bioanalytical method which is simple, rapid and precise with the economically available mobile phase in combined dosage form.

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2. MATERIALS AND METHODS

2.1Materials and Instruments

Shimadzu HPLC with LC solutions software is used for the simultaneous estimation which is equipped with the PDA detector. AR and HPLC grade of solvents like formic acid, methanol, acetonitrile and milliQ water is utilized for the analysis of the drugs. API (Diclofenac sodium and Tolperisone Hydrochloride) are received as a gift samples from Alomone labs, Israel.

2.2 Determination of Purity of API

The purity of the Diclofenac sodium and tolpersione Hydrochloride was determined by FT-IR(Fourier –Transform Infra red spectroscopy) and DSC (Differential Scanning Calorimetry).The FT –IR spectra is characterized within the

range of 4000-400cm^{-1} at a spectral resolution of 2 cm^{-1}.On similar lines, thermograms are recorded at a ambient temperature up to 400°C.

2.3 Preparation of diluent

A prepared combination of methanol (90%) and 0.1% formic acid in water at the ratio of 90:10.The combined mixture has been degassed in a ultra sonic water bath for 15 minutes and finally filtered through 0.22µ membrane filter.

2.4 Preparation of Diclofenac Sodium standard stock solution

About 10mg of Diclofenac sodium was weighed and transferred to 10mL of volumetric flask. The volume is made up to 10mL of diluent to prepare 1mg/mL of stock solution.

2.5 Preparation of Tolperisone standard stock solution

To prepare 1mg/mL of stock solution, about 10mg of tolperisone hydrochloride is weighed and transferred to 10mL volumetric flask and the volume is made up to 10mL with diluent.

2.6 Preparation of Sample solution for Assay

Tolperitas (Batch No. TSD 306, Intas pharmaceutical limted, Ahmedabad, India) containing diclofenac sodium (50mg) and tolperisone hydrochloride (150mg) is used. 10 tablets are crushed and the contents are dissolved in methanol followed by centrifugation at 6000rpm. Supernatant liquid is collected and methanol is evaporated by rota dryer. Residual moisture is removed by lyophilization.API obtained is checked for purity by FT IR and DSC. API is stored at 2-8°C in airtight container for further use. To prepare 1mg/mL of stock solution, 10mg of tolperisone hydrochloride and diclofenac sodium mixture has been weighed and transferred to 10mL volumetric flask, volume which is made up to 10mL diluent.

2.7 Preparation of system suitability stock solution

Weigh accurately 10mg of diclofenac sodium and 30mg of tolperisone hydrochloride at working standard and transfer it to 10mL volumetric flask. Add 5mL of diluent and sonicated to dissolve the contents. Finally the volume is prepared to 10mL using diluent.

2.8 Chromatographic Methods [3]

The chromatographic parameters used are summarized in table 1. For system suitability, the mixed standard solutions has been injected for 3 times and the corresponding chromatograms are recorded .In similar lines, placebo solution, accuracy, LOD, LOQ, linearity, specificity, robustness and ruggedness is also been recorded. All the peaks are recorded in the wavelength of 255 nm. On contrary, robustness and ruggedness are analyzed by using the varied wavelengths at 250 & 260 nm.

2.9 Assay

20µL of the sample and standard is injected in to the chromatographic system and the measured areas of tolperisone and diclofenac sodium peaks are determined and the percentage of purity is calculated by using the formula:

% of purity = AT/AS x WS/ DS x DT/ WT x P/100 x Avg.wt/ Label claim x 100

Where :

AT = average area of sample

AS = average area of standard

WS = weight of working standard taken in mg

WT = weight of sample taken in mg

DS = Dilution factor of standard solution

DT = Dilution factor of sample solution

P = Percentage purity of working standard

LC = Label claim of tolperisone and diclofenac sodium.

2.9.1 Plasma spiking studies [4]

Interference of matrix effect on developed RP – HPLC was determined by spiking known concentrations of tolperisone and diclofenac sodium in rat plasma. Rat plasma is collected and filtered through 0.22µm filter. Known concentrations of diclofenac sodium and tolperisone are spiked and incubated for 6 hours. The plasma proteins are precipitated using methanol, followed by centrifugation at 5000 rpm. Methanol fraction is collected and purified by solid phase extraction cartridges (Oasis C18). Samples are concentrated and analyzed by RP – HPLC method. Linearity study of diclofenac sodium (1-10µg/mL) and tolperisone (3-30µg/mL) is performed and the percentage recovery of diclofenac sodium and tolperisone has been determined and recorded in Table 2

3.0 RESULTS AND DISCUSSION

3.1 Determination of Purity of diclofenac sodium and Tolperisone

The purity of diclofenac sodium and tolperisone is determined by FT –IR and DSC. FT –IR studies shows characterstic peaks of tolperisone observed at 2944 cm^{-1} (CH stretching aromatic hydrogen), 1200-1600 cm^{-1} (C=C stretching of benzene), 2955 cm^{-1} (CH stretching) and 1678 cm^{-1} (Carbonyl stretching) and diclofenac sodium shows the characteristic peaks at 3413 cm^{-1} (N-H stretching),3630 cm^{-1} (OH stretching), 3080 cm^{-1} (CH aromatic hydrogen stretching) and 1652 cm^{-1} (Carbonyl stretching). The spectra has been illustrated in Fig.1.

DSC studies shows the characteristic endotherm of tolperisone and diclofenac sodium at 187° C and 285° C respectively (Fig.2).

3.2 Method development and optimization of chromatographic parameters

The method developed for the analysis of diclofenac sodium and tolperisone is a sensitive, accurate, precise HPLC method. To optimize the mobile phase, various combinations of buffer, acetonitrile and methanol is studied using C18 column (250X4.6mm). Initially, the combination of acetonitrile : water (90:10) is tried for the separation of diclofenac sodium and tolperisone in combined dosage form. But the resolution of the peaks are not satisfactory. Hence the mobile phase containing methanol: water (70:30) has been found that the peaks resolute with higher retention time and tailing. In order to overcome this unsatisfied resolution, the mobile phase containing a mixture of methanol and water in the ratio of 90:10 and was acidified with 0.1 % formic acid. The resolute peaks appeared in good shape and showed times at 2.5 and 4.2 min respectively. (Fig.3).

3.3 Analytical method validation

Validation of an analytical method is the process to establish by laboratory studies that the performance characteristic of the method meets the requirements for the intended analytical application. Performance characteristics is expressed in terms of analytical parameters.

3.3.1 Accuracy

The accuracy of the method shall be demonstrated through determination on samples in three concentrations from 80% (3µg/mL and 9µg/mL of diclofenac sodium and tolperisone respectively), 100% (4µg/mL and 12µg/mL of diclofenac sodium and tolperisone respectively), 120% (5µg/mL and 15µg/mL of diclofenac sodium and tolperisone respectively). Samples are analyzed in triplicate using optimal conditions of RP –HPLC method and chromatograms are recorded (Table 3; Fig.4).

3.3.2 Method precision

Precision is studied by injecting standard solutions of diclofenac sodium and tolperisone mixture (6µg/mL and 18µg/mL respectively) for six times on same day (Intra- day study) and repeated on the second day (inter day study). The chromatograms are recorded and % RSD is calculated (Table 4 & 5; Fig. 5-6)

3.3.3 Reproducibility (Intermediate precision)

Intermediate precision of the method is studied by analyzing the corresponding responses three times on the same day and on different days for three different concentrations of standard solutions of tolperisone (12µg/mL, 18µg/mL and 24µg/mL) and diclofenac sodium (4µg/mL, 6µg/mL and 8µg/mL). The chromatograms are recorded (Fig. 10-11) and % RSD are tabulated in the Table 6 &7.

3.3.4 Limit of detection (LOD)

The detection limit of an individual analytical procedure is at lowest amount of analyte in a sample which can be detected but not necessarily recommended as exact value. The standard mean values for diclofenac sodium and tolperisone are 2854 and1325 respectively. The LOD values of diclofenac sodium and tolperisone are 0.57µg/mL and 0.85µg/mL respectively.

3.3.5 Limit of quantitation (LOQ)

The quantitation limit of an analytical procedure is at the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The LOQ values for diclofenac sodium and tolperisone are 1.74µg/mL and 2.58µg/mL respectively.

3.3.6 Linearity

The linearity is performed by injecting 20µL of standard solutions of diclofenac sodium (1-10µg/mL) and Tolperisone (3-30µg/mL) for three times and the chromatograms are recorded in Fig 10 and tabulated in Table 8.

3.3.7 Robustness and Ruggedness

Robustness and ruggedness has been studied in the samples of diclofenac sodium and tolperisone by deliberate change in the wavelength (+/-5nm) and column temperature (+/-5°C). It has been observed that there is no change in the RT and area of samples which illustrates the good robustness of the developed method.

3.3.8 Plasma spiking studies

Linearity of diclofenac sodium and tolperisone is performed in the presence of rat plasma. It is observed that quantitative linearity was obeyed in the concentration range of 1-10µg/mL and 3-30µg/mL for diclofenac sodium and tolperisone respectively. The regression equations of concentration over their peak areas are found to be Y=16521x (R^2 = 0.9974), Y =5156.4x (R^2 = 0.9994) for diclofenac sodium and tolperisone respectively where Y is the peak area and X is concentration of drugs in rat plasma (µg/mL). The mean percentage recoveries in plasma for tolperisone are 98.4+/-1.50, 99.7+/-0.8 and 98.3+/-1.3 at 80%, 100% and 120% levels. The mean percentage recoveries in plasma for diclofenac sodium are 99.4+/-0.6,99.8+/-1.6 and 99.8+/-0.5 at 80%, 100% and 120% levels. The data has been tabulated in Table 2 and Illustrated in Fig 7-9.

Table 1: Optimized chromatographic and validation parameters

Optimization parameters	Results	Acceptance range
Chromatographic parameters		
Stationary phase	C18 column, 150X4.6mm	NA
Mobile phase composition	90:10 of Methanol:0.1% Formic acid	NA
Detection wavelength	255nm	NA
Injection volume	20µl	NA
Column temperature	25±2˚C	NA
Flow rate	1 Ml/min	NA
Run time	10 Min	NA
Validation parameters		
Linearity	DFS linearity range is 1-10µg/Ml, TOL linearity range is 3-30µg/Ml	$R^2 \geq 0.99$, similar response ratios
LOD	DFS and TOL were 0.57µg/ml and µg/ml 0.85 respectively	S/N Ratio 3:1
LOQ	DFS and TOL were 1.74 µg/ml and 2.58 µg/ml respectively	S/N ratio 10:1
Precision	The % RSD values of TOL and DFS were found to be 0.10 and 0.13 respectively	RSD<2%
Specificity	Retention time of TOL, DFS were 2.56 min, 4.21 min respectively.	No interference
Accuracy	The mean percentage recoveries for TOL were 99.30±2.50, 100.50±1.50 and 99.30±2.50 at 80%, 100% and 120% levels. The mean percentage recoveries for DFS were 99.30±1.50, 99.80±1.00 and 100.10±1.0 at 80%, 100% and 120% levels	98-102%,

Table 3: Accuracy

Level	TOL	DFS	TOL			DFS		
	Area	Area	Actual amount added	Recovery	% Recovery	Actual amount added	Recovery	% Recovery
80%	47249	49076	9	9.18	102	3	2.97	99
	45862	50067	9	8.91	99.01	3	3.03	101
	44935	48580	9	8.73	97.01	3	2.94	98
				Average	99.30		Average	99.30
				STDEV	2.50		STDEV	1.50
100%	62993	65438	12	12.24	102	4	3.96	99
	61149	66731	12	11.88	99.01	4	4.04	101
	61993	65762	12	12.05	100.38	4	3.98	99
				Average	100.50		Average	99.80
				STDEV	1.50		STDEV	1.00
120%	78749	81794	15	15.3	102.01	5	4.95	99
	76432	83446	15	14.85	99.0	5	5.05	101
	74888	82968	15	14.55	97.0	5	5.02	100
				Average	99.30		Average	100.10
				STDEV	2.50		STDEV	1.00

Table 4: Intra day method of precision

Intra-day precision	TOL		DFS	
	RT	Area	RT	Area
18µg/ml:6µg/ml	2.56	43672	4.21	91862
	2.56	43672	4.21	91847
	2.56	43686	4.21	91899
	2.56	43684	4.22	91846
	2.56	43672	4.22	91800
	2.56	43667	4.22	91827
Average	2.56	43676	4.21	91847
STDEV	0.001	5.635	0.003	3.283
% CV	0.05	0.02	0.08	0.04

Table 5: Inter day method of precision

Inter day precision	TOL		DFS	
	RT	Area	RT	Area
18µg/ml:6µg/ml	2.56	43669	4.21	91865
	2.56	43672	4.21	91859
	2.56	43676	4.21	91869
	2.56	43685	4.22	91865
	2.56	43679	4.22	91875
	2.56	43676	4.22	91867
Average	2.56	43677	4.22	91866
STDEV	0.001	5.56	0.01	5.28
% CV	0.001	0.01	0.13	0.01

Table 6: Reproducibility – Intra day

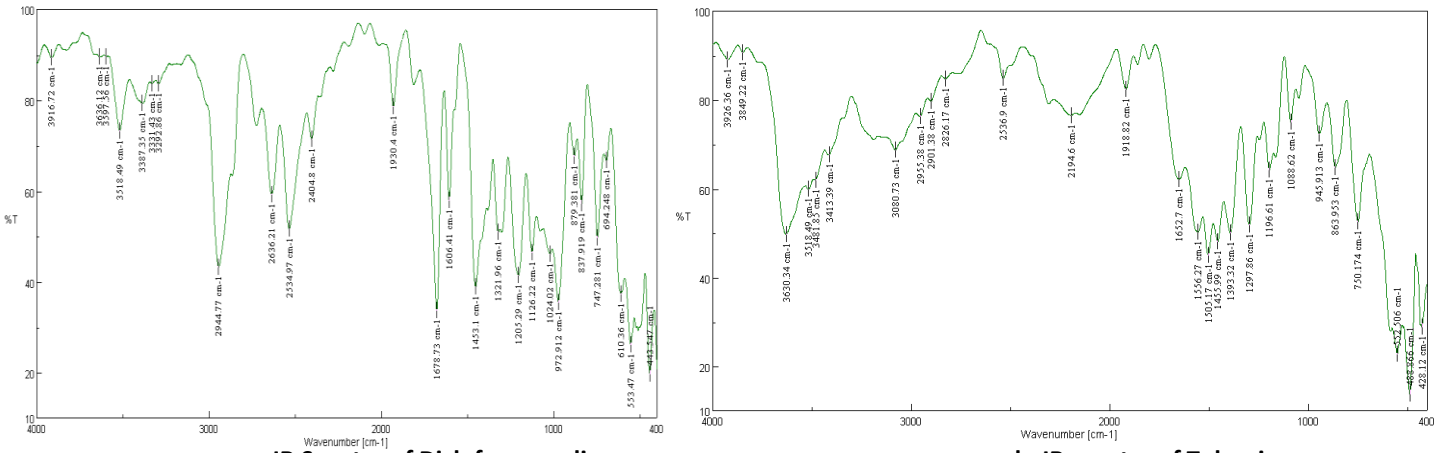
	TOL		DFS		TOL		DFS		TOL		DFS	
	RT	**Area**	**RT**	**Area**	**RT**	**Area**	**RT**	**Area**	**RT**	**Area**	**RT**	**Area**
	12µg/ml:4µg/ml				**18µg/ml:6µg/ml**				**24µg/ml:8µg/ml**			
Inter day	2.57	37663	4.213	60869	2.56	46574	4.214	88774	2.56	58782	4.212	123572
	2.57	37659	4.215	60874	2.56	46578	4.213	88771	2.56	58779	4.209	123570
	2.57	37665	4.214	60871	2.56	46569	4.213	88768	2.56	58778	4.212	123569
Avg	2.57	37662	4.214	60871	2.56	46574	4.2133	88771	2.56	58780	4.211	123570
Stdev	0.00	3.06	0.00	2.52	0.00	4.51	0.00	3.00	0.00	2.08	0.00	1.53
% CV	0.00	0.01	0.02	0.00	0.00	0.01	0.01	0.00	0.00	0.00	0.04	0.00

Table 7: Reproducibility – Inter day

	TOL		DFS		TOL		DFS		TOL		DFS	
	RT	**Area**	**RT**	**Area**	**RT**	**Area**	**RT**	**Area**	**RT**	**Area**	**RT**	**Area**
	12µg/ml:4µg/ml				**18µg/ml:6µg/ml**				**24µg/ml:8µg/ml**			
Inter day	2.57	37661	4.213	60869	2.56	46578	4.214	88774	2.56	58780	4.21	123568
	2.57	37660	4.215	60874	2.56	46575	4.213	88771	2.56	58777	4.21	123562
	2.57	37664	4.214	60871	2.56	46573	4.213	88768	2.56	58774	4.21	123561
Avg	2.57	37662	4.214	60871	2.56	46575	4.2133	88771	2.56	58777	4.211	123564
Stdev	0.00	2.08	0.00	2.52	0.00	2.52	0.00	3.00	0.00	3.00	0.00	3.79
% CV	0.00	0.01	0.02	0.00	0.00	0.01	0.01	0.00	0.00	0.01	0.04	0.00

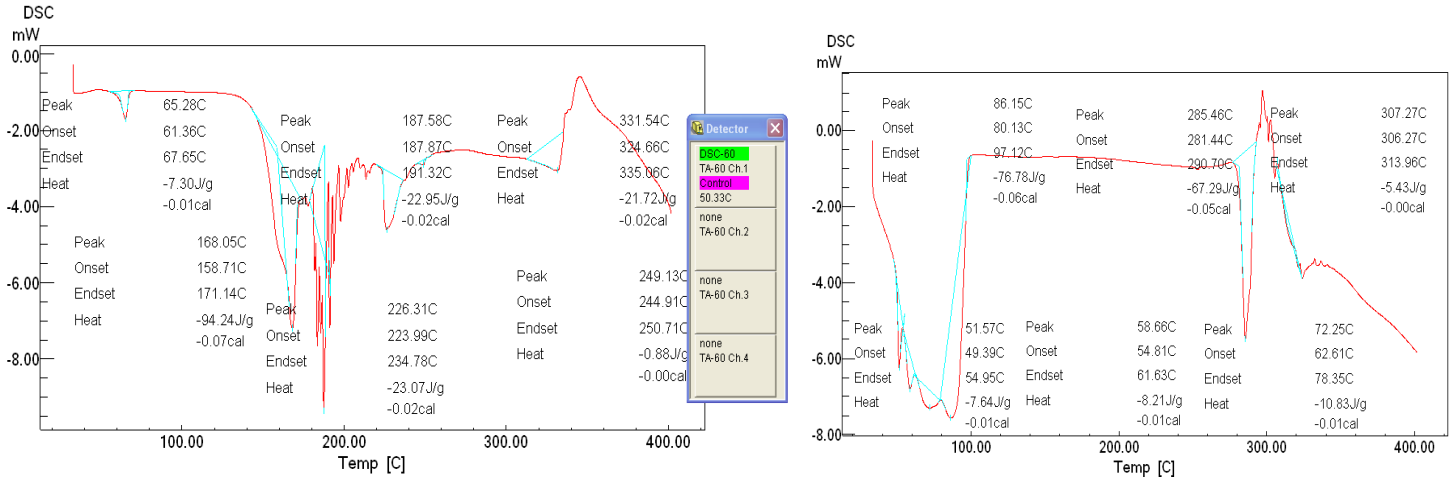
Table 8: Linearity

Drug	Conc.(µg/ml)	Equation of regression line	R^2
TOL	3-30	Y = 5146.7x	0.9993
DFS	1-10	Y = 16524x	0.9974



a. IR Spectra of Diclofenac sodium **b. IR spectra of Tolperisone**

Figure 1: IR Spectra of diclofenac sodium and tolperisone



a. Diclofenac sodium **b. Tolperisone**

Figure 2: DSC thermograms of diclofenac sodium and tolperisone

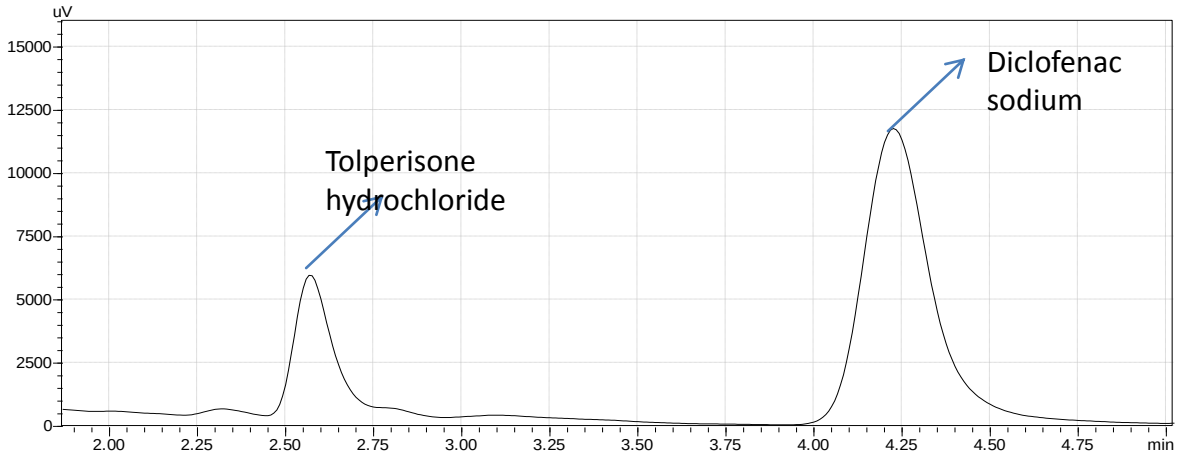


Figure 3: Chromatographic optimization

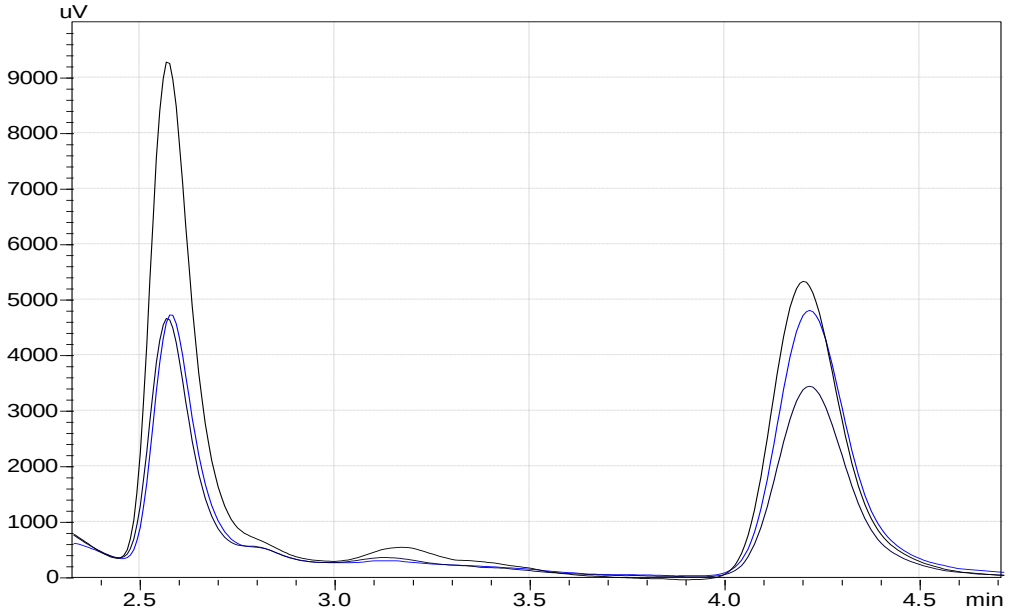


Figure 4: Overlay chromatogram of accuracy

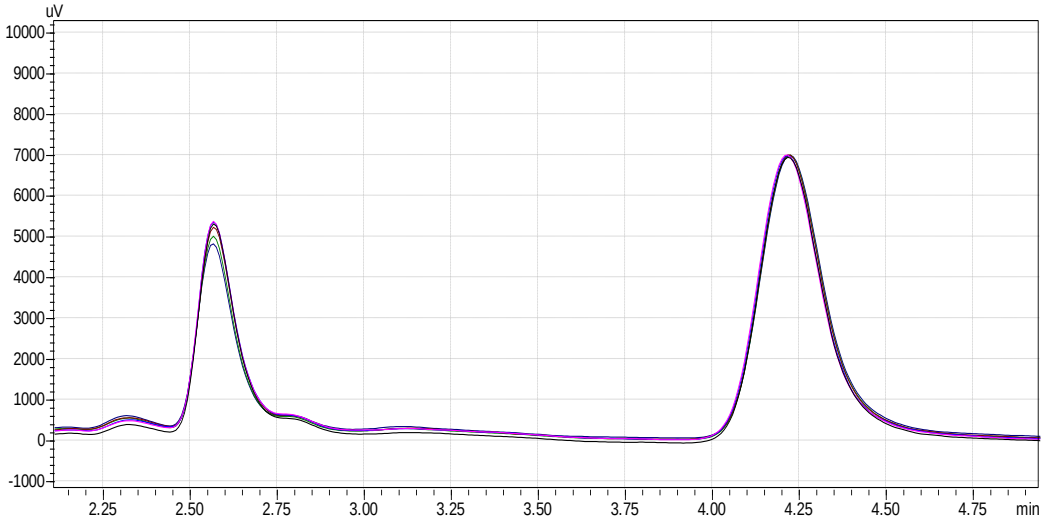


Figure 5: Overlay chromatogram of Intra day precision

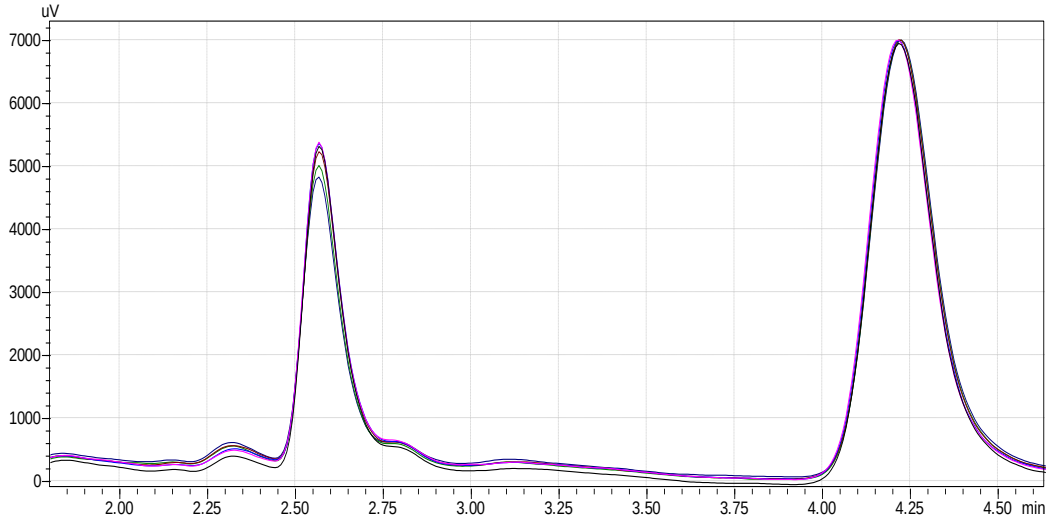
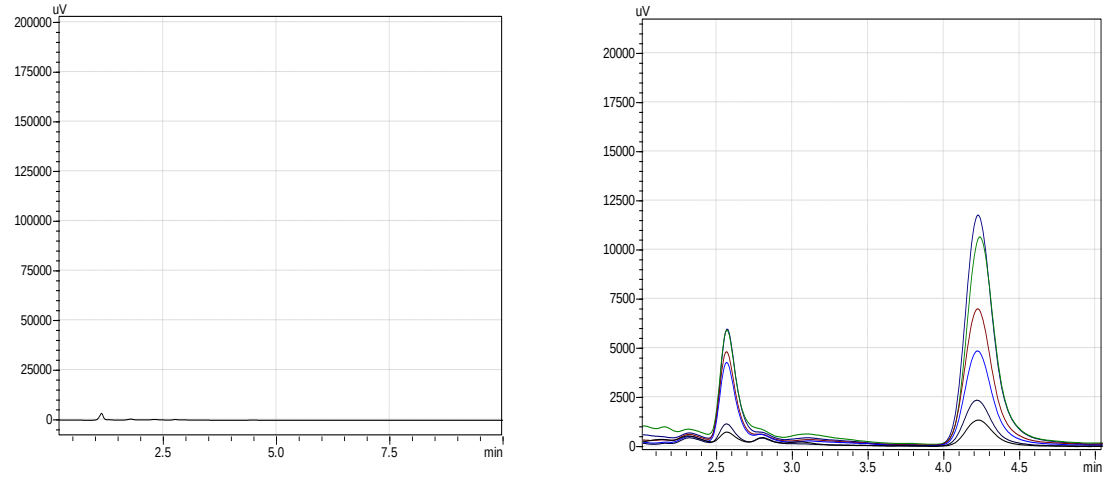


Figure 6: Overlay chromatogram of Inter day precision



(a) (b)

Figure 7: Representative chromatogram of rat plasma after purification by solid phase extraction (A) and Overlain chromatogram of linearity of DFS and TOL in Rat plasma (B).

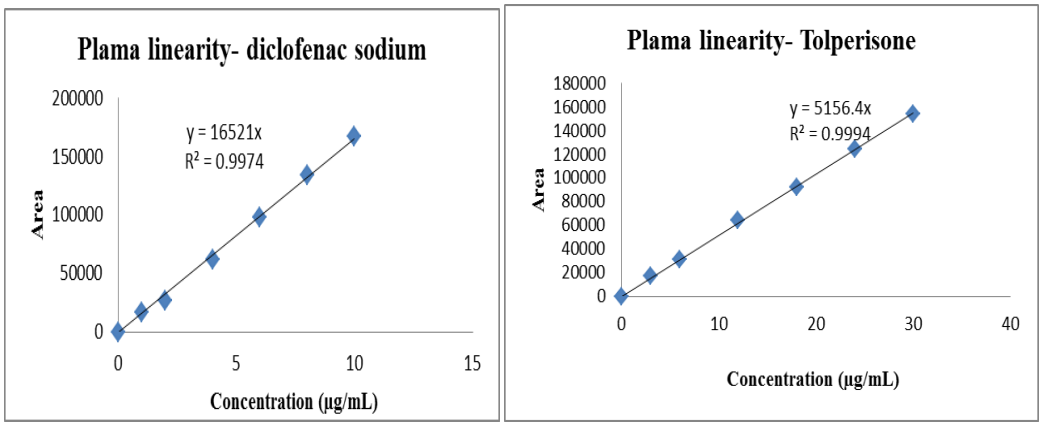


Figure 8: Linearity of DFS (A) and TOL (B) in Rat plasma

4. CONCLUSION

The present work reports that a new RP- HPLC method for the simultaneous estimation of tolperisone hydrochloride and diclofenac sodium are in bulk and pharmaceutical dosage form. This developed method is optimized by developing the combination of methanol and water in the ratio of 90:10 acidified with 0.1% formic acid. It resulted in the good resolution of peaks with good shape with a flow rate of 1Ml/min. The retention times of tolperisone and diclofenac sodium are found to be 2.5 and 4.2 min respectively. As per the ICH guidelines, quantitative linearity has obeyed in the concentration range of 1 to 10µg/Ml, 3 to 30µg/Ml for diclofenac sodium and tolperisone respectively. The regression equations of concentration over their peak areas has been found to be Y = 16524x (R^2 = 0.99) and Y = 5146.7 x (R^2 = 0.99) for diclofenac sodium and tolperisone respectively. Further it is found that no interference of external matrix when the drugs have been spiked in rat plasma. It is observed that 98% recovery of tolperisone hydrochloride and diclofenac sodium is possible with this developed method. Hence this method is a simple, sensitive, precise and accurate for the simultaneous estimation of tolperisone hydrochloride and diclofenac sodium in bulk and pharmaceutical dosage form.

5. ACKNOWLEDGEMENT

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