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Green Bio Estimation of Co-Administered Non-Steroidal Anti Inflammatory Drug Diclofenac and Leflunomide in Rat Plasma by New HPLC Method

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Abstract

An eco-friendly HPLC- DAD method is introduced for the bioanalysis of co-administered non-steroidal anti-inflammatory drug diclofenac and leflunomide in rat plasma. Leflunomide and NSAIDs are commonly prescribed in combination This method aimed to estimate the co-adminstered leflunomide and diclofenac combination for their simultaneous separation and quantification. The method was conducted with gradient elution of a mobile phase composed of ethanol and phosphate buffer with a flow rate of 1 mL/min over a Zorbax Eclipse Plus-C18 (4.6 × 250 mm × 5 µm) column. The drug peaks were picked using a diode array detector at 275 nm. The developed method was validated according to ICH guidelines in terms of accuracy, precision, linearity, range, LOD and LOQ. The concentration range was found to be linear in the range of 2 – 60 µg/mL. The LOD and LOQ values were found to be very small (0.16, 0.47 µg/mL and 0.22, 0.68 µg/mL) for Diclofenac and Leflunommide respectively. The % RSD and the % R were found within the acceptable range. The method was applied for *in vitro* and *in vivo* analysis of diclofenac and leflunomide with mean recoveries of 97.5 to 102.30 and 97.31 to 101.20 respectively.

Keywords: *Green method; bio analysis; NSAID; leflunomide.*

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are one of the most consumed drugs worldwide either by prescription or over-the-counter and are used for symptomatic treatment of chronic inflammatory diseases like rheumatoid arthritis, osteoarthritis, and gout and the relief of acute pain conditions like headache, postoperative pain, and orthopedic fractures [1,2]. The basic mode of action of NSAIDs is inhibition of the pro-inflammatory cyclooxygenase (COX) enzyme, which is responsible for the conversion of arachidonic acid, a fatty acid present in cell membranes, to inflammatory prostanoids (prostaglandins, prostacyclin, and thromboxane) [2,3].

Leflunomide is an immunomodulatory drug used to treat moderate to severe rheumatoid arthritis. Leflunomide is a pro-drug that is rapidly and completely converted in the gut wall and liver to its active metabolite, teriflunomide A771726. The latter exerts its anti-inflammatory effects by inhibiting the mitochondrial enzyme dihydroorotate dehydrogenase (DHODH), which plays a key role in the de novo synthesis of the pyrimidine ribonucleotide uridine monophosphate (RUMP) required for the synthesis of DNA and RNA thus interferes with the synthesis of immune cells. Leflunomide is commonly prescribed with NSAID as a combination for the treatment of rheumatoid arthritis [4]. Figures 1A and 1B show the structural formula of the studied drugs [5]. The increasing demand for NSAIDs and immunomodulators makes the need for an analytical method for their green estimation an urgent issue. The studied drugs are leflunomide (LEF) and diclofenac (DIC).

Reversed-phase high-performance liquid chromatography (RP-HPLC) is the most widely used analytical technique in pharmaceutical analysis. The

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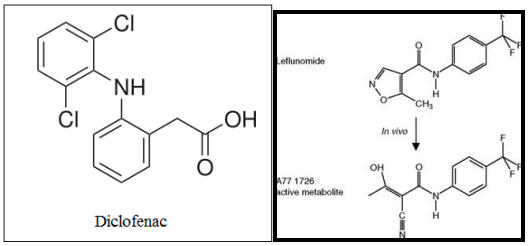
mobile phase of RP-HPLC is usually a igmixture of water containing additives to adjust pH and ionic strength and organic solvents, such as acetonitrile (ACN) and methanol (MeOH). These two solvents are noticeably the preferred organic solvents used in RP-HPLC because of their exceptional and convenient properties in terms of good miscibility with water, the low viscosity of their aqueous solutions, low UV cut-off wavelengths (190 nm for ACN and 205 nm for MeOH), availability of high purity HPLC grades and significantly inert with most samples and HPLC column [6,7].



A B

Fig.1: (A) Structural formula of the studied NSAID's (B) Structure of leflunomide and its active metabolit

However, ACN and MeOH bring up some concerns in terms of environmental impact and health safety that cannot be neglected due to the large amounts consumed in RP-HPLC methods that generate high quantities of waste to be disposed of. Both solvents are considered hazardous due to their inherent toxicity and great requirements for their waste disposal. However, MeOH is considered more environmentally friendly as it is less toxic and more biodegradable [8]. In this context, green HPLC methods are becoming preferable. Some strategies are being practiced to achieve greener liquid chromatography methods. One strategy is to replace the classically used organic solvents (i.e., acetonitrile and methanol) with eco-friendlier alternatives. So far, ethanol has been the most used alternative organic solvent. Other strategies were involved, such as the use of totally aqueous mobile phases, micellar liquid chromatography, and ionic liquids where these approaches have been well developed, as they do not require equipment investments and are rather economical [5]. Furthermore, other strategies have commonly been implemented that focus on reducing the amount of solvent consumed by decreasing column length, internal diameter, and/or particle size thus decreasing the amount of toxic waste generated, however, they require the purchase of expensive ultra-high-performance liquid chromatography (UHPLC) instruments [9,10].

Literature search reveals that numerous methods were reported for the individual assays of these drugs and also for their combination. Attempts have been made to develop a method for the determination of several NSAIDs together in different biological matrices and pharmaceutical formulations [11-18] or in water samples [19-24]. Other methods reported the simultaneous determination of NSAIDs in presence of co-prescribed drugs [25-26]. Only one of the above-reported methods was environmentally friendly where they used 0.1% formic acid and methanol (30:70) as mobile phase for the simultaneous assay of nonsteroidal anti-inflammatory drugs (NSAIDs) including indoprofen, ketoprofen, naproxen, diclofenac, ibuprofen, mefenamic acid, and tolfenamic acid [23]. Several HPLC trials were made for the determination of LEF alone or in the presence of its metabolite [27-30], or in plasma [31,32]. In addition, the determination of the metabolite in plasma, serum, and urine was reported in some studies [33-37]. Simultaneous determination of LEF and NSAIDs in formulations and biological fluids were also reported [38,39]. Lastly, Stability indicating methods were reported for LEF using RP-HPLC methods [40-42], spectrophotometric method [43], and TLC densitometric methods [44]. So far no HPLC method has been reported for the simultaneous determination of the mentioned NSAIDs in combination with LEF. Furthermore, the literature search confirmed that there is no eco-friendly RP-HPLC method reported for the simultaneous estimation of LEF and the aforementioned NSAID in serum. Therefore, the present work aimed to develop a simple, sensitive, accurate, and eco-friendly RP-HPLC method for the simultaneous separation and quantification of LEF and diclofenac NSAID in serum.

2.Experimental

2.1. Instrumentation

The chromatographic separation was achieved using HPLC-DAD Agilent 1200 series (auto-injector, quaternary pump, vacuum degasser and diode array, and multiple wavelength detector G1315 C/D and G1365 C/D) connected to a computer loaded with

Agilent Chem. Station Software (Agilent Technologies, Santa Clara, CA, USA).

Separation of the analytes was accomplished using a reversed-phase column Zorbax Eclipse Plus-C18 column (4.6 × 250 mm × 5µm). A deuterium lamp was used as a source of continuous UV radiation over the 190 - 400 nm range. pH measurements were made using a digital pH meter 3310 Jenway.

2.2. Materials and reagents

Leflunomide and diclofenac were kindly supplied by Evapharm, and Pharco pharmaceutical companies respectively. All chemicals used were analytical grade reagents. HPLC-grade. Ethanol and diethyl ether were purchased from Fisher Scientific (Leicestershire, UK). Methanol was obtained from Alpha Chemika, India. HPLC-grade dibasic potassium hydrogen phosphate was bought from Sigma-Aldrich (Japan). Orthophosphoric acid (85%) was obtained from S.D. Fine Chemicals Limited, India. Sodium hydroxide and HCl (37%) were purchased from El-Nasr Chemical Co.

Stock solutions of the studied drugs (1mg/mL) were prepared in ethanol. Working solutions were obtained by further dilution of the stock solution in the same solvent. One molar solution (1M) of both hydrochloric acid and sodium hydroxide was also prepared in distilled water.

2.3. Chromatographic condition

Zorbax Eclipse Plus-C18 (4.6 × 250 mm × 5 µm) column was used as a stationary phase. A mobile phase consisted of 0.005 M phosphate buffer solution (pH 5) and ethanol was pumped at a flow rate of 1 mL/min with gradient elution as shown in table 1. The eluent was monitored by the diode array detector (DAD) from 200 to 400 nm and the chromatogram was extracted at the wavelength of 275 nm. All measurements were performed at 30℃.

Table 1: Gradient program used in the proposed HPLC-DAD method.

Time (min)	0.005 M PO_4 Buffer %	Ethanol %
0	60	40
2	60	40
12	40	60
15	40	60

2.4. General procedure and construction of calibration graphs

Accurate volumes of LEF and DIC standard stock solutions were transferred into a set of 10 mL volumetric flasks and diluted to volume with ethanol to reach the concentration range (2 – 60 µg/mL). Triplicate 5 µL injections were made for each concentration and chromatographed under the previously described HPLC conditions. The peak areas were plotted against the corresponding concentrations to construct the calibration curves.

2.5. Analysis of leflunomide and diclofenc in spiked rat plasma

Seven adult male albino rats weighing from 280 to 300 g were used in this study. They were housed in clean cages with proper ventilation under the same environmental conditions and allowed free access to food and water throughout the study. Animals were allowed a two-week pre-experimentation period to be acclimatized to the laboratory conditions**.** Blood samples were obtained from the orbital sinus of the rats under light ethyl ether anesthesia, with capillary tubes [45]. The spurting blood was collected in clean and sterile blood sample collection tubes and allowed to clot for 30 min at room temperature, then centrifuged at a rate of 5,000 revolutions per minute (rpm) for 10 min at 4 °C. The serum obtained was separated and transferred into clean Eppendorf tubes for re-centrifugation at a rate of 15,000 rpm for 10 min at 4 °C then the clear serum was separated and labeled to be used in the assay of spiked serum.

2.5.1. Procedure in serum

Into clean dry small test tubes, aliquots of 0.5 mL of serum were accurately transferred and spiked with different concentrations of LEF and DIC. Five microliters of ACE drug stock solution were added as an internal standard. Twenty-five microliters of 0.1 N HCl were added and solutions were mixed using a vortex shaker. The mixtures were extracted with 3 mL of diethyl ether. The solutions were further mixed using a vortex tube shaker, then centrifuged at 4000 rpm for 5 minutes, and finally frozen at -80 °C for 2 hr till the aqueous layers at the bottom of the test tubes became solid. The clear supernatant ether layers were transferred into other clean, dry test tubes and left for evaporation under nitrogen gas. The remaining residues were reconstituted in 0.5 mL of mobile phase (0.25 mL ethanol and 0.25 mL phosphate buffer) and the resulting solutions were filtered with a 0.45 µm syringe filter, then injected into the HPLC and chromatographed using the proposed method.

2.6. In vivo assay of DIC and LEF in rat serum

Single dose solutions of DIC and LEF were administered to 6 male Albino rats by oral route using a BD syringe with an oral gavage needle (size 18) at the dose of 10 mg/kg and 4 mg/kg body weight for DIC and LEF, respectively. Approximately 1.5 mL blood samples were collected from each anesthetized rat at a pre-determined time interval using a capillary tube into a pre-labeled blood sample collection tube. Sample collection was made after approximately 15 min. The procedure was then performed under analysis of leflunomide and diclofenac in spiked rat plasma.

3. Results and discussion

3.1. Optimization and method development

Several experiments were carried out to optimize the chromatographic method for the separation of leflunomide with the NSAID diclofenac drug with acceptable peak symmetry within a relatively short analysis time. For stationary phase optimization, Zorbax Eclipse XDB-C18 (4.6 × 150 mm × 5 µm) column was tried and resulted in an overlap of a few peaks and after trying to reach a more acceptable separation by adjusting the pH of the phosphate buffer and the percentage of organic solvent, the peaks suffered from broadening and tailing. On the other hand, Zorbax Eclipse Plus-C18 (4.6 × 250 mm × 5 µm) column produced the best-resolved peaks where sharp and symmetric peaks were achieved subsequently, it became the column of choice for this assay. Since the aim of the study was to develop an eco-friendly method, therefore acetonitrile and methanol were excluded from the beginning and only ethanol was tried. The type of buffer was tested by using 0.1 % formic acid, acetate buffer, and phosphate buffer at different pH values. The eluents suffered from tailing and long retention times in the case of using 0.1% formic acid and acetate buffer. The peaks became more symmetrical in the case of phosphate buffer. The effect of phosphate buffer pH was studied within the range of 2.5 to 7 by altering the pH of the aqueous phase. At pH 3.5, peaks suffered from fronting and tailing, at pH 4.5, at pH 5.5, peaks were asymmetric and tailed with poor resolution, at pH 6 and 7 the peaks overlapped.

The resolution and peak symmetry was enhanced upon adjusting the pH to 5 but the peaks still eluted at long retention times. The ionic strength of phosphate buffer was investigated by changing the concentration of dibasic potassium hydrogen phosphate over the range 0.003M to 0.007M. Upon increasing the buffer concentration, the retention times were slightly increased and the noise increased also. So, the buffer concentration was reduced to reduce the analysis time but some peaks overlapped. The optimum concentration which enhanced the resolution and shorten the retention was 0.005 M. Moreover, the isocratic elution of different proportions of 0.005 M phosphate buffer and ethanol did not provide adequate baseline separation between all peaks. Additionally, it caused longer retention times and poorly resolved asymmetric peaks, therefore, gradient elution was considered. The gradient elution started with higher aqueous and lower organic modifier ratios to ensure adequate separation between the early eluting peaks, then the organic modifier ratio was increased linearly up to a certain value to allow separation of the remaining peaks in reasonable retention times. Several gradient systems were tested and the best resolution with shorter retention times and symmetrical peaks was fulfilled using a gradient system that starts with 40% (by volume) ethanol maintained for 2 min and increased linearly to 60% at 12 min then maintained at this percentage till the end of the run (Table 1). 1 mL/min was the best flow rate at 30 ºC as a baseline was stable.

Quantification was done at 275 nm by measuring of peak area and recording the spectra of eluted peaks using DAD. The examined drugs showed broad absorption bands over the range of 200 - 400 nm with maximum absorption of around 260 nm for LEF and around 280 nm for DIC. A wavelength of 275 nm was chosen since it possesses good absorbance of both analytes.

The chromatographic conditions described above showed excellent separation of the analytes, DIC at 11.82 min, and LEF at 13.70 min. Analytical parameters for the determination of DIC and LEF in serum using the proposed HPLC-DAD method are shown in table 2.

Table 2: Analytical parameters for the determination of DIC and LEF respectively by HPLC

Parameter \ Drug	Diclofenac (DIC)	Leflunomide (LEF)
Concentration range (µg/mL)	2 - 60	2 - 60
Intercept (a)	0.31	-28.22
S_a	3.71	6.35
Slope (b)	13.65	17.45
S_b	0.12	0.20
RSD% of the slope (S_b%)	0.85	1.14
Correlation coefficient (r)	0.99967	0.99941
$S_{y/x}$	6.84	11.67
F value	13755.02	7681.52
Significance F	1.21×10^{-15}	1.66×10^{-14}
LOD (µg/mL)	0.16	0.22
LOQ (µg/mL)	0.47	0.68

3.2. Application to spiked rat serum samples

The developed method was used for the analysis of DIC and LEF in biological fluids such as rat serum. The previously discussed procedure in serum was implemented. The peak areas of both drugs were divided by the peak areas of the IS to get the response ratio or the response factor RF which will be used in the regression analysis of the results. Under the optimized conditions described above, the measured response ratios were found to be proportional to the concentrations of each drug. Regression analysis for the calibration curves, demonstrated good linearity over the concentration ranges of 0.5 – 50 and 0.25 – 20 µg/mL for DIC and LEF, respectively. This was confirmed by the correlation coefficient values > 0.99 as shown in Table 2. Moreover, acceptable results were obtained by the % recoveries that ranged from 97.50 to 102.3 and 97.31 to 101.20% for DIC and LEF respectively. Representative chromatograms showing the separation of both spiked drugs DIC and LEF together with the internal standard (IS) by the proposed method are displayed in Figure 2. Additionally, peak purity was confirmed by recording the UV absorption spectrum at several points across each peak which was found pure and homogenous without any interference from serum. The matrix effect was studied by three replicates analysis of six different rat plasma spiked with low and high concentrations of DIC (0.5, 50 µg/mL) and LEF (0.25, 20 µg/mL). The plasma peak and the compounds peaks were well resolved and % recoveries were found to be accepted with values ranging from 97.33 to 101.71 and from 97.50 to 101.88 for DIC and LEF, respectively. The Carry-over effect was evaluated by injection of blank plasma samples after calibration, the obtained chromatograms showed no observed peaks of the DIC or LEF at 12.098 and 13.624, respectively.

Application to in vivo assay

The *in vivo* assay results revealed that the proposed method applies to the assay of DIC and LEF in serum samples obtained from rats after giving them the drugs orally. The % found for each drug is demonstrated for each rat separately in Table 3. The chromatogram showing the separated drugs from the *in vivo* assay is shown in Figure 3. Finally, the appearance of an additional peak at a retention time of 9.15 min with the spectrum shown in Figure 3, was found to have a similar spectrum to teriflunomide; the active metabolite of LEF. A reference to this metabolite spectrum was found in a work done by Sharma P. et al [46,47]. This suggests that our proposed method is also capable of analyzing both the raw drug and its metabolite simultaneously which will be studied in future work.

3.3 Analytical Validation

The proposed method were validated as per the International Conference on Harmonization guidelines (ICH) [7].

3.3.1. Linearity and concentration ranges

The proposed method was appraised for linearity by analyzing a series of different concentrations for both DIC and LEF drugs. The linear regression equations were generated by the least-squares treatment of the peak areas versus the corresponding concentrations. Under the optimized conditions described above, the measured peak areas were found to be proportional to the concentrations of each drug. After performing regression analysis for the calibration curves, good linearity was demonstrated over the concentration

ranges of 2 – 60 µg/mL for both DIC and LEF drugs that was further confirmed by the correlation coefficient values > 0.999 with RSD% of slope values (S_b %) less than 2%. Table 2 shows the linear regression equations, concentration ranges, correlation coefficients, standard deviation of the intercept (S_a) and slope (S_b), the variance ratio (F) and standard deviation of residuals ($S_{y/x}$). The latter is considered an important statistical parameter as it indicates the degree of random error in the estimated "y" values. The smaller the standard error of the estimate the closer the points are to the straight line.

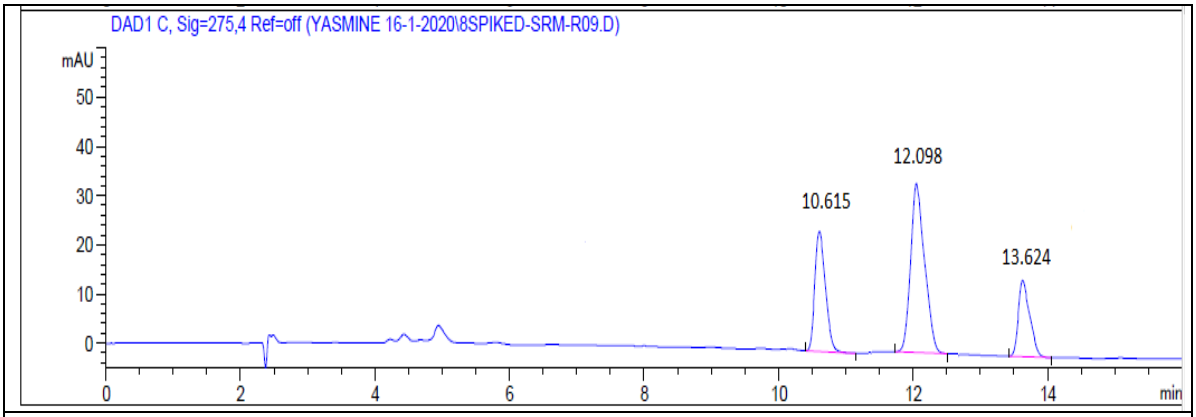


Figure 2: HPLC chromatogram for spiked plasma with IS

1: ACE (t_R=10.615) 2: DIC (t_R= 12.098) 3: LEF (t_R=13.624)

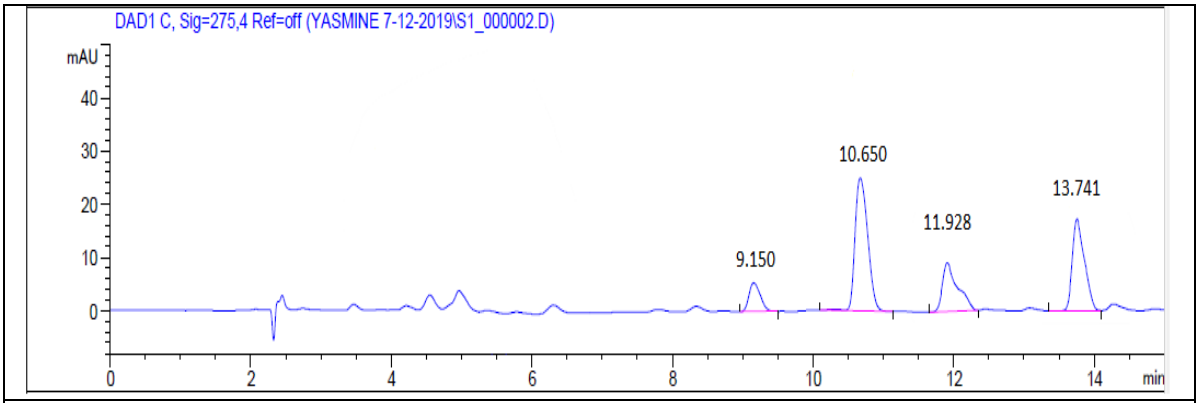


Figure 3: HPLC chromatogram of *in vivo* sample

Table 3. Application of the proposed methods to the analysis of dosage form

Parameters	LEF	DIC
%Found ± SD*	99.82±0.54	100.40±0.26

*Mean ± standard deviation for six determinations.

3.3.2. Limit of detection (LOD) and limit of quantitation (LOQ)

The limit of detection (LOD) is the lowest concentration of the analyte that can be detected but not necessarily quantitated under the applied experimental conditions while the limit of quantitation (LOQ) is the lowest concentration that can be determined with acceptable precision and accuracy. Both the LOD and LOQ were calculated according to the ICH guidelines. LOD is defined as the concentration of the analyte which has a signal-to-noise ratio of 3:1 while the LOQ, the ratio required is 10:1. They are given in Table 2

3.3.3. Accuracy and Precision

According to the ICH guidelines, accuracy and precision should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range [7]. Consequently, the accuracy and within-day (intra-day) precision for the proposed method were studied at three concentration levels for each drug within the studied linearity ranges with three replicate determinations for each concentration. Concentrations studied were 10, 30, 50 µg/mL for both drugs. Similarly, the

accuracy and between-day (inter-day) precision were tested by analyzing the same three concentrations for each drug using three replicate determinations repeated on three days. The recovered concentrations were calculated using the corresponding regression equations. Accuracy and precision are expressed as percentage relative error (E_r %) and percentage relative standard deviation (RSD %) summarized in Table 4. The values of E_r % and RSD % did not exceed 2% which reflect the high precision and good accuracy of the developed method for the estimation of analytes in their bulk form.

Table 4: Precision and accuracy results for the analysis of DIC and LEF mixture using the proposed HPLC-DAD.

		Nominal value (µg/mL)	Found ± SD* (µg/mL)	RSD (%)	E_r (%)
DIC	Within-day	2	9.84 ± 0.03	0.29	-1.56
		30	29.82 ± 0.29	0.96	-0.60
		60	49.61 ± 0.43	0.88	-0.78
	Between-day	2	9.96 ± 0.17	1.67	-0.43
		30	29.93 ± 0.44	1.46	-0.24
		60	49.79 ± 0.64	1.29	-0.41
LEF	Within-day	2	9.87 ± 0.08	0.8	-1.33
		30	30.40 ± 0.34	1.12	1.35
		60	50.03 ± 0.74	1.47	0.06
	Between-day	2	9.95 ± 0.11	1.16	0.44
		30	30.07 ± 0.50	1.65	0.25
		60	50.24 ± 0.89	1.78	0.48

*Mean ± standard deviation for three determinations.

3.3.4. Specificity

The specificity of the proposed HPLC method was investigated by testing the spectral purity of the eluted peaks for the 2 analytes. The results indicated that the eluted peaks are pure. Additionally, specificity was further demonstrated by the separation of the analytes from their stress degradation products.

3.3.5. Robustness

Robustness was evaluated by performing small variations in different conditions such as buffer pH (± 0.2 unit), flow rate (± 0.1 mL/ min), working wavelengths (± 2 nm), column temperature (± 3 °C) and the percentage of the organic solvent in the mobile phase (± 5% of ethanol in the mobile phase). These variations had no significant effect on the retention times of the eluting peaks and the measured responses (peak areas).

3.3.6. System suitability

System suitability parameters including retention times (t_R), capacity factors (k′), number of theoretical plates (N), symmetry, selectivity (α) and resolution (R_s) were studied. The %RSD were calculated for each parameter and the values were found to be less than 2%.

3.3.7. Stability of solutions

The stability of standard solutions was examined over 24 hr. at room temperature. The solutions remained unchanged with no sign of degradation.

4. Method greenness assessment

Although, the chromatographic methods of analysis of drugs are the most common but they have adverse effectson the environment as they might generate toxic and carcinogenic waste. The greenness of our method was evaluated according to PBT (persistency, bioaccumulation, toxicity), corrosive, hazardous, and waste. The utilized ethanol, water, and buffer were assed as green chemicals (El-Yazbi et al., 2020). So, the proposed analytical method can be considered an eco-friendly method.

Conclusion

A simple, fast, sensitive, eco-friendly, and accurate reverse phase HPLC method has been described for the determination of NSAID diclofenac drug and leflunomide. The chromatographic methods are the most commonly used for determination of drugs due to good analytical performances in addition to the greenness of the method which make it environmentally friendly method. The method was straightforward and simpler than the commonly used HPLC methods involving ion pair or derivatization. It

was also clear from the chromatograms that both the active ingredient peaks in all the stress conditions were free from any sort of degradation impurities. This method would be suitable for the stability studies, cleaning validation, and routine analysis of pharmaceutical dosage forms in quality control and R&D laboratories for products of similar type and composition.

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