

Development of Liquid Chromatography – UV Method for Simultaneous Determination of Leflunomide and NSAIDs in API and Pharmaceutical Formulations: It's Application to *In vitro* Interaction Studies

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Abstract

An efficient analytical method for the simultaneous determination of leflunomide and non steroidal anti-inflammatory drugs in API and formulations by LC-UV has been developed. The analytes were separated on Purospher Star, C₁₈ (5 µm, 250×4.6 mm) column at ambient temperature with methanol: water (80:20, v/v, pH at 2.7) at flow rate of 1.5 mL min⁻¹. Experiment was conducted in two phases. Leflunomide was separated with flurbiprofen and ibuprofen (phase-I) and diclofenac sodium and mefenamic acid (phase II). Calibration curves were linear over the range 0.625–5 µg mL⁻¹ in both phases for leflunomide while for flurbiprofen, ibuprofen, diclofenac sodium, mefenamic acid linearity were achieved in the range of 0.625–5, 11.25–90, 1.56–50 and 0.78–25 µg mL⁻¹, respectively with *r*²>0.9998. Intra-day variation was <1.2 and <1.4 %, while in inter-day ranged between 0.042–1.45% and 0.08–1.27% in phase-I and II, respectively. Mean recovery values for intra-day ranged from 99.04–100.4% and 98.48–100.2% and for inter-day were between 98.54–100.29% and 98.85–100.54% in phase-I and II, respectively. The LLOD of leflunomide was 13 ng mL⁻¹, while LLOQ was 39ng mL⁻¹, respectively. LLOD and LLOQ for flurbiprofen, ibuprofen, diclofenac sodium and mefenamic acid were 6.9, 296, 71 and 1.2 ng mL⁻¹ and 21, 897, 214.3 and 3.676 ng mL⁻¹, respectively. Present study showed that nanogram quantities of all the compounds can be estimated accurately. The newly established method was successfully applied to study *in vitro* interactions between leflunomide and NSAIDs.

Keywords: Leflunomide; Ibuprofen; Flurbiprofen; Diclofenac sodium; Mefenamic acid; HPLC; Interaction

Introduction

Leflunomide (Figure 1), 5-methyl-*N*-[4-(trifluoromethyl) phenyl]-isoxazole-4-carboxamide is a leading disease modifying anti-rheumatic drug (DMARD) to treat rheumatoid arthritis (RA) [1]. Leflunomide and the malononitriloamides (MNA) are a new class of immunomodulating drugs that have been investigated for use in transplantation. Anti-inflammatory and immunomodulating properties of leflunomide were recognized in 1985, which differ from classical anti-inflammatory and immunosuppressive drugs. Leflunomide has a long half-life (11 to 16 d) in humans, and because of this its clinical development has been restricted to use in patients of rheumatoid arthritis [2].

In phase II and III clinical trials of active rheumatoid arthritis, leflunomide was shown to improve primary and secondary outcome

measures with a satisfactory safety profile. The active metabolite of leflunomide, A77 1726, at low, therapeutically applicable doses, reversibly inhibits dihydroorotate dehydrogenase (DHODH), the rate limiting step in the de novo synthesis of pyrimidines. Continuing research indicates that A77 1726 may down regulate the glycosylation of adhesion molecules, effectively reducing cell-cell contact activation during inflammation [3]. It inhibits T-lymphocyte proliferation after converting to its active metabolite i.e. A771726 in human to produce its anti-arthritis action [4-6].

Leflunomide is a prodrug that is rapidly converted in the gastrointestinal tract and plasma to its active, open ring metabolite, the malononitrilamide, A771726 (2-cyano- 3-hydroxy-*N*-(4-trifluoromethylphenyl) butenamide). Structure-activity studies have shown how modifications to A771726 affect its immunoregulatory activity [7-9]. This drug along with NSAIDs (prostaglandin inhibitors) is used in the initial stages of remedy within three months of diagnosis to eradicate or diminish soreness, irritation, joint damage and to sustain normal function in RA patient [10,11].

As leflunomide and NSAIDs are commonly prescribed in combination, the objective of this study was to establish an efficient, reliable, accurate and sensitive method for their simultaneous separation and quantification. Previously, numerous methods were developed

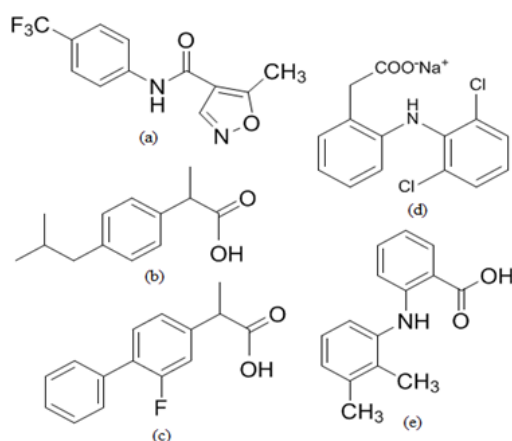


Figure 1: Chemical structures of (a) leflunomide, (b) flurbiprofen (c) ibuprofen, (d) diclofenac sodium and (e) mefenamic acid.

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and validated for leflunomide determination and quantification in IP, dosage formulations [12,13] and serum [14-16]. We have reported determination of leflunomide by RP-HPLC [17]. A method of reversed phase chromatography has been reported to separate seven NSAIDs i.e. naproxen, ketoprofen, ibuprofen, diclofenac, piroxicam, nimesulide and paracetamol using benzoic acid as an internal standard. Detection was made by two detectors, one by UV where compounds were detected at 245 nm and another by electrospray-mass spectroscopy where except paracetamol all NSAIDs were detected by negative ionization mode. Experiment was conducted by isocratic mode using acetonitrile–water with 0.1% acetic acid as the mobile phase [18].

A number of methods for the simultaneous determination of co-administered drugs have also been reported by our research group as simultaneous determination of rosuvastatin, lisinopril, captopril and enalapril [19], lisinopril, [20] verapamil [21], captopril [22], ceftriaxone sodium [23], diltiazem [24] rosuvastatin [25] and sparfloxacin [26] with NSAIDs in API, pharmaceutical formulations and human serum by RP-HPLC. Arayne et al. [27-34] and Sultana et al. [35-40] have previously reported, a number of methods for drug-drug interaction studies using UV-visible spectroscopic and RP-HPLC. In continuation of this work we have attempted to study *in vitro* interactions of leflunomide with flurbiprofen, ibuprofen, diclofenac sodium and mefenamic acid (Figure 1), the experiment was accomplished by the use of UV-visible spectroscopic and RP-HPLC techniques. For this purpose, interactions were studied at human environmental conditions pH 4 and 9 where maximum quantity of leflunomide remains in its original form, while at pH 7.4 (pH of the blood), its metabolite form, malononitrilamide, A77 1726 (2-cyano-3-hydroxy-*N*-(4-trifluoromethylphenyl) butenamide) is present in maximum concentration [4]. No liquid chromatographic method has yet been reported for separation and simultaneous analysis of leflunomide along with more than one NSAID although these drugs are co-administered simultaneously, so we have developed a simple, reliable, accurate, sensitive, cost effective and least time consuming method suitable for the simultaneous analysis of leflunomide and NSAIDs in API and pharmaceutical formulations. This method has been applied successfully to interaction studies of leflunomide with NSAIDs.

Experimental

Materials

Leflunomide (reference standard) was a gift from Hilton Pharma, reference standards of all the NSAIDs were supplied by Lab-9 of the Department of Chemistry, University of Karachi. Pharmaceutical formulations leflunomide (Lefora 10mg, 20mg), flurbiprofen (Synalgo[®] 100 mg), ibuprofen (Dolofen 200 mg tablet), diclofenac sodium (Voltral[®] 50 mg) of Novartis Pharma and mefenamic acid (Ponstan[®] 250 mg) of Parke-Davis & Co Ltd were purchased from local Pharmacy. Each product was labeled and expiry date not earlier than two years, at the time of these studies.

All reagents were of HPLC grade. Methanol and phosphoric acid (85%) (Merck, Germany) and HPLC-grade. Ultra-purified filtered water was used to prepare the mobile phase (80:20 (v/v) methanol–water).

Chromatographic conditions

The apparatus used for analysis consisted of Shimadzu model LC-10AT VP pump with a SPD-10AT VP, variable wavelength UV-visible detector and chromatographic system was integrated via Shimadzu model CBM-102 Communication Bus Module. Simultaneous determination of leflunomide with NSAIDs was conducted in two

phases. In the phase-I, leflunomide was simultaneously determined and validated with flurbiprofen and ibuprofen and in phase-II, it was determined along with diclofenac sodium and mefenamic acid. In both phases analysis were carried out by using methanol–water (80:20 v/v) at pH 2.7 on Hiber, RT 250–4.6 Purospher Star RP-18 endcapped (5 μ m) column at flow rate 1.5 mL min⁻¹. Detection was made at 254 nm. Before delivering into the system, it was filtered through a 0.45 μ m millipore filter and degassed in an ultrasonic bath. The sample volume of 10 μ L was injected through a rheodyne injector valve into HPLC system.

General procedure

Stock solutions of leflunomide and NSAIDs were prepared by dissolving the drugs in methanol to yield concentration of 100 μ g mL⁻¹. These solutions were prepared once and stored at 4°C protected from light. Calibration standards were prepared by diluting the stock solutions in the range of 0.625–5 μ g mL⁻¹ for both phases for leflunomide while for flurbiprofen, ibuprofen, diclofenac sodium, mefenamic acid solutions were diluted in the range 0.625–5, 11.25–90, 1.56–50 and 0.78–25 μ g mL⁻¹, respectively. 20 μ L of these solutions were injected into the LC system (n=5). Before analysis, all the solutions were filtered through a 0.45 μ m vacuumed filter and degassed by sonicator.

Analysis of pharmaceutical formulation

Ten tablets of each formulation were separately powdered and amount equivalent to 10mg of leflunomide, flurbiprofen, ibuprofen, diclofenac sodium and mefenamic acid were dissolved in mobile phase in separate 100 mL volumetric flasks. The solutions were subjected to vigorous shaking and then allowed to stand for 1 h with intermittent sonication for complete extraction of the drug. All the solutions were then filtered and volume was brought to mark with mobile phase and then treated as above.

Procedure for leflunomide-NSAIDs interactions

In this phase of experiments, stock solutions (100 μ g mL⁻¹) of leflunomide and interacting NSAIDs (flurbiprofen, ibuprofen, diclofenac sodium and mefenamic acid) were prepared in buffers of pH 4 (pH of full stomach) and pH 9 (simulated intestinal juice) individually. These two pH were selected for studying leflunomide *in vitro* interactions because at these pH, leflunomide remains in its original form, while at pH 7.4 (pH of the blood), its metabolite, malononitrilamide, A77 1726 (2-cyano-3-hydroxy-*N*-(4-trifluoromethylphenyl) butenamide) is present in maximum concentration [5,6]. In case of leflunomide, first it was dissolved in minimum quantity of methanol and then diluted by buffer of pH 4 and pH 9 individually. These solutions were mixed in equimolar ratios in Erlenmeyer flasks and refluxed at 37 \pm 5°C for two hours. An aliquot of 5 mL was withdrawn after every 30 minutes interval for two hours. After appropriate dilutions, aliquots were filtered via 0.45 μ filter paper and analyzed by RP-HPLC method. Concentration of each drug was determined using linear equation and percentage availability was calculated. The data was analyzed by student t-test using p<0.05 as significant value.

The above aliquots of interacting drugs were also analyzed by using UV-spectrophotometer and data was also analyzed by student t-test using p<0.05 as significant value.

Results and Discussion

Optimization of the chromatographic conditions

RP-HPLC is a high-ranking tool for drug analysis; the advantages of short retention time, method reliability, sensitivity and drug specificity substantiate the use of HPLC for various groups of drugs. Therefore the

main objective of this study was to develop a simple, easy and effectual liquid chromatographic method with UV detection for simultaneous analysis of leflunomide and NSAIDs in bulk drug and pharmaceutical formulations. Initially C₁₈ Discovery column (125 cm×4.6 mm, 5 µm particle) and Phosphor Start (5 µm, 12.5×0.46 mm) analytical reverse-phased column were used for separation of leflunomide and NSAIDs. Due to poor resolution and high retention time (more than 15 minutes), these columns were not selected for this work. Use of Hiber, RT 250-4.6 Purospher Star RP-18 end capped (5 µm) column produced good symmetrical peaks with high resolution and short retention time.

In case of solvent selection, isocratic mode was applied for elution instead of gradient to avoid re-equilibration [24]. Initially, method was optimized by varying methanol: water ratios (80:20, 85:15 v/v) at various pH (3.4, 3.2, 2.9, 2.7) and flow rates (0.8 mL.min⁻¹, 1.2 mL.min⁻¹, 1.5 mL.min⁻¹) which lead to considerable changes in the chromatographic parameters, like peak symmetry, drug resolutions and retention time. However, the ratio of methanol: water in 80:20 (v/v) with flow rate 1.5 mL.min⁻¹ at pH 2.7 at 254 nm yielded best results. It had been observed (Figures 2a and 2b) that drugs were best separated and gave well-shaped narrow peaks at low pH and selected flow rates. Alteration in both parameters also affected the retention times for all drugs while excellent result was achieved at pH 2.7 ± 0.2 keeping flow rate 1.5 ± 0.2 mL.min⁻¹.

Peak identification

Under optimized conditions, the peaks of leflunomide and mentioned NSAIDs were identified by comparing chromatograph of leflunomide and NSAIDs standards with the chromatograph of their tablets (Figures 2a and 2b). Under described chromatographic conditions, in phase-I, leflunomide, flurbiprofen and ibuprofen were eluted with retention times of 2.9, 3.37 and 3.8 minutes, respectively while in phase -II leflunomide, diclofenac sodium and mefenamic acid were eluted with retention times of 2.9, 3.6 and 4.9 minutes, respectively.

Validation

Importance of validation for a developed method to check its suitability for intended purpose holds prime position. The developed method was validated according to ICH guidelines [25] and USP 2002 [26]. It includes various parameters as system suitability, selectivity, specificity, linearity, accuracy, precision (robustness and ruggedness) and sensitivity which were followed to validate the method.

System suitability

To assure the appropriate work of the method during whole analysis,

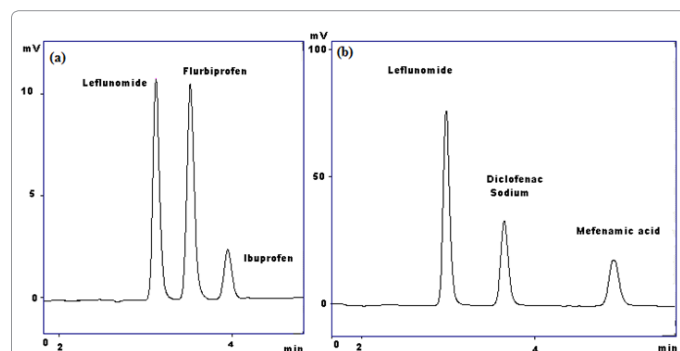


Figure 2: (a) Phase-I Leflunomide (2.9 min) chromatogram with flurbiprofen (3.37 min) and ibuprofen (3.8 min) and (b) Phase-II. Leflunomide (2.9 min) chromatogram with diclofenac sodium (3.6 min) and mefenamic acid (4.9 min).

system suitability were checked by injecting six replicates of each drug standard solutions and appraisal was made by analyzing repeatability, relative retention, column efficiency (number of theoretical plate), capacity factor and symmetry factor as indicated in Table 1.

Linearity

For linearity, linear regression analysis was performed using Microsoft Excel 2003 software. Calibration curves were found to be linear over the range of 0.625–5 µg mL⁻¹ in both phases for leflunomide while for flurbiprofen, ibuprofen, diclofenac sodium, mefenamic acid linearity were achieved in the range 0.625-5, 11.25-90, 1.56-50 and 0.78-25 µg mL⁻¹, respectively with correlation coefficients (*r*²) of >0.9998 (Table 2). For leflunomide, minimum limit of analyte that the method can detect i.e. detection limits (LLOD) was calculated by the formula LLOD=3.3 SD/ slope and in pharmaceutical formulation it was found 13 ng mL⁻¹, respectively. Quantification limit (LLOQ) was evaluated as ten times the noise level (LLOD=10 SD/ slope) and were found as 39 ng mL⁻¹, respectively. LLOD and LLOQ for flurbiprofen, ibuprofen, diclofenac sodium and mefenamic acid were 6.9, 296, 71 and 1.2 ng mL⁻¹ and 21, 897, 214.3 and 3.676 ng mL⁻¹, respectively, suggesting that nanogram quantities of all the compounds can be estimated accurately (Table 3).

Precision and accuracy

For precision intra-day (repeatability) and inter-day (reproducibility) analysis were performed which are expressed as the coefficient of variation (RSD=standard deviation/mean*100). %RSD is a useful parameter to compare uncertainty between different measurements of varying absolute magnitude. For this purpose six concentrations (n=6) of each drug in the linear range were analyzed on the same day (intra-day precision) and two consecutive days (inter-day precision). Accuracy of the method was determined as percentage recovery of known amount of these drugs in their dosage solution. Intra-day % RSD values of the measurements of all analytes ranged between 0.17–1.15 % in phase-I while 0.02 – 1.35 % in phase-II while in inter-day % RSD ranged between 0.042–1.45 % in phase-I and 0.08–1.27 % in phase-II. The mean recovery values for intra-day were 99.04–100.4% in phase-I and 98.48–100.2% in phase-II while for inter-day ranged between 98.54–100.29% in phase-I and 98.85–100.54% in phase-II, as indicated in Table 2.

Selectivity and sensitivity

This method was selective and sensitive. In the representative chromatograms (Figures 3a and 3b) of the drugs in formulation there was no extra peak reflecting no interference of excipients present.

Stability

The stability of standard and sample solutions of leflunomide was evaluated by assay after 24 h at 4°C against fresh standard solutions, which showed that leflunomide was stable and did not show significant variations in the time span of 24 hours.

Ruggedness

Method ruggedness was performed by two analysts on separate lots of leflunomide. Each analyst prepared samples in triplicate and used separate instruments, reagents and mobile phase solutions. % RSD (n=5) for all of the samples for each lot was less than 3.5 % and method did not show any notable deviation in results from acceptable limits.

Robustness

Deliberate changes were made by altering the flow rate, pH and

Analytes	Retention time (T _R) (min)	Capacity factor (K')	Theoretical plates (N)	Tailing factor (T)	Resolution (R)	Separation factor (α)
pH 2.7 ± 0.05						
<i>Phase-1</i>						
Lef	2.9 ± 0.024	0.54 ± 0.013	7182 ± 0.58.2	1.26 ± 0.09	1.26 ± 0.14	2.68 ± 0.06
Flur	3.37 ± 0.21	0.75 ± 0.101	7606 ± 0.37.9	1.18 ± 0.093	1.18 ± 0.07	1.37 ± 0.13
Ibu	3.8 ± 0.18	0.96 ± 0.021	9769 ± 53.1	1.17 ± 0.17	1.17 ± 0.10	1.29 ± 0.21
<i>Phase-2</i>						
Lef	2.9 ± 0.08	0.41 ± 0.051	7229 ± 53	1.21 ± 0.11	1.21 ± 0.15	3.9 ± 0.15
Diclo	3.6 ± 0.23	0.73 ± 0.019	7735 ± 78	1.15 ± 0.07	1.15 ± 0.084	1.78 ± 0.05
Mef	4.9 ± 0.15	1.34 ± 0.09	8629 ± 23	1.14 ± 0.13	1.14 ± 0.17	1.82 ± 0.29
Flow rate 1.5 ± 0.2						
<i>Phase-1</i>						
Lef	2.91 ± 0.13	0.54 ± 0.08	7181 ± 67	1.25 ± 0.13	1.262 ± 0.05	2.68 ± 0.21
Flur	3.367 ± 0.16	0.75 ± 0.051	7619 ± 25	1.182 ± 0.07	1.19 ± 0.12	1.37 ± 0.07
Ibu	3.81 ± 0.25	0.96 ± 0.13	9771 ± 89	1.20 ± 0.15	1.16 ± 0.11	1.29 ± 0.061
<i>Phase-2</i>						
Lef	2.9 ± 0.13	0.41 ± 0.087	7230 ± 33	1.21 ± 0.16	1.21 ± 0.18	3.9 ± 0.22
Diclo	3.6 ± 0.098	0.73 ± 0.12	7743 ± 27	1.15 ± 0.59	1.15 ± 0.12	1.78 ± 0.09
Mef	4.9 ± 0.14	1.34 ± 0.21	8631 ± 76	1.14 ± 0.2	1.14 ± 0.19	1.82 ± 0.13
Methanol percentage 80 ± 2%						
<i>Phase-1</i>						
Lef	2.89 ± 0.21	0.539 ± 0.07	7183 ± 83	1.26 ± 0.06	1.21 ± 0.051	2.66 ± 0.08
Flur	3.36 ± 0.31	0.752 ± 0.052	7616 ± 34	1.18 ± 0.09	1.15 ± 0.21	1.37 ± 0.31
Ibu	3.81 ± 0.09	0.95 ± 0.12	9760 ± 67	1.17 ± 0.14	1.14 ± 0.17	1.30 ± 0.16
<i>Phase-2</i>						
Lef	2.91 ± 0.19	0.41 ± 0.05	7243 ± 49	1.21 ± 0.11	1.20 ± 0.08	3.9 ± 0.07
Diclo	3.56 ± 0.22	0.73 ± 0.061	7727 ± 33	1.15 ± 0.097	1.14 ± 0.019	1.78 ± 0.25
Mef	4.89 ± 0.071	1.34 ± 0.074	8632 ± 92	1.14 ± 0.13	1.21 ± 0.13	1.82 ± 0.31

Lef=leflunomide, Flur=flurbiprofen, Ibu=ibuprofen, Mef=mefenamic acid, Diclo=diclofenac sodium. All data presented in mean ± standard deviation (n=6)

Table 1: System suitability and ruggedness of the developed method.

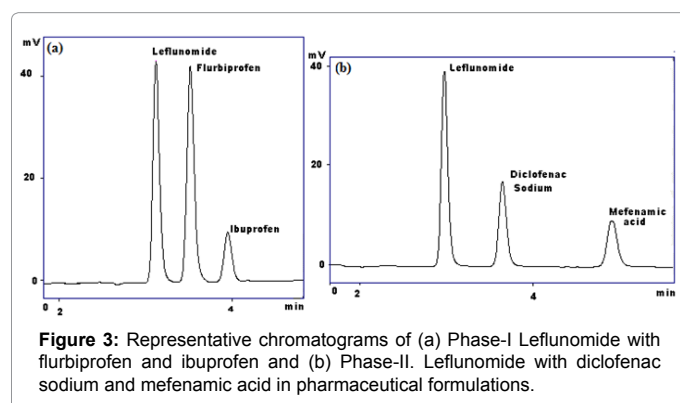
Drugs	Conc (µg mL ⁻¹)	Intraday precision		Interday precision	
		% Rec	% RSD	% Rec	% RSD
Phase-1					
Lef	0.625	99.88	0.168	99.57	0.102
	1.25	100.12	0.102	99.54	0.103
	1.875	99.85	1.15	99.9	0.85
	2.5	100.05	1.06	100.11	1.45
	5	99.19	1.14	99.66	0.74
Flur	0.625	100.26	0.39	100.29	0.58
	1.25	100.07	0.53	100.04	0.39
	1.875	99.04	0.087	99.98	1.02
	2.5	100.01	0.192	99.94	0.042
	5	99.61	0.48	100.2	1.08
Ibu	11.25	100.41	1.12	99.97	0.166
	22.5	100.02	1.02	98.54	0.32
	33.75	100.10	0.24	100.2	1.08
	45	99.76	0.29	99.89	0.33
	90	99.42	0.17	100.12	0.90
Phase-2					
Lef	0.625	99.78	0.34	99.57	0.08
	1.25	99.88	0.06	99.54	0.27
	1.875	100.02	0.02	100.29	0.29
	2.5	99.57	0.03	100.18	0.87
	5	100.48	0.63	99.66	0.06
Diclo	1.56	99.46	1.13	100.16	0.18
	6.25	100.21	0.26	99.87	0.29
	12.5	100.5	0.15	99.98	1.17
	25	98.78	0.66	100.2	0.68
	50	100.1	0.63	99.85	1.27
Mef	0.78	98.78	0.28	100.1	0.015
	3.125	100.03	0.86	100.54	0.97
	6.25	100.18	1.35	98.98	0.016
	12.5	99.78	0.37	99.48	0.99
	25	98.48	0.14	100.2	0.43

Table 2: Precision and recovery of leflunomide and NSAIDs.

Drugs	Regression equation	R2	LLOD ng mL ⁻¹	LLOQ ng mL ⁻¹
Phase-1				
Lef	y=11428x + 1345.6	0.9998	13	39
Flur	y=13149x + 118.64	0.9998	6.9	21
Ibu	y=204.9x – 970.43	0.9985	296	897
Phase-2				
Lef	y=16783x + 7014.6	0.998	13	39
Diclo	y=4488.5x – 369.24	0.9992	71	214.3
Mef	y=2766.6x + 11798	0.9985	1.2	3.676

Lef=leflunomide, Flur=flurbiprofen, Ibu=ibuprofen, Mef=mefenamic acid, Diclo=diclofenac sodium (n=6)

Table 3: Sensitivity of the proposed methods.



composition of the mobile phase to validate the robustness of the method. Data (Table 4) indicates that there was no significant variation while making these changes.

Interaction studies of Leflunomide with NSAIDs by UV and HPLC techniques

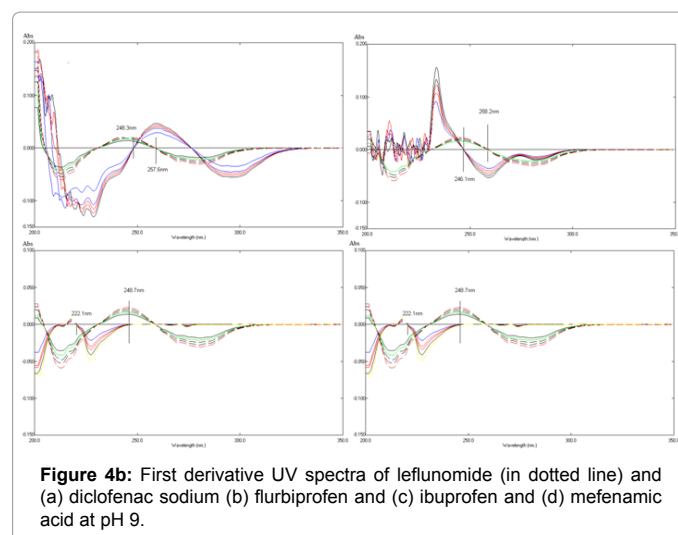
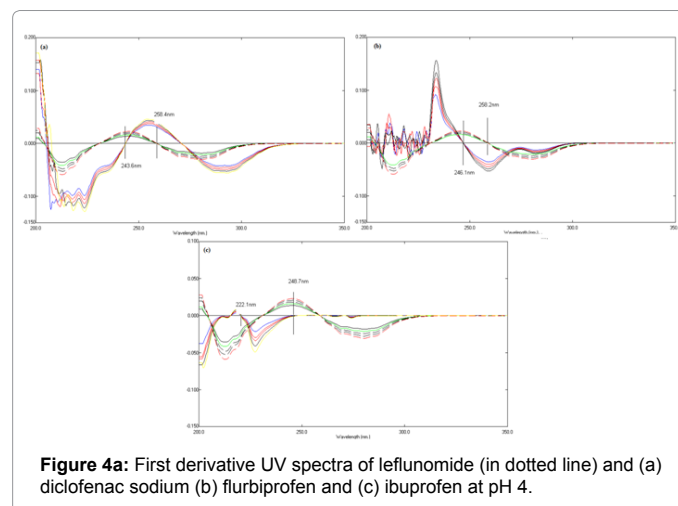
UV- spectrophotometer: The UV/visible spectra (zero-order) of leflunomide in presence of NSAIDs in buffers of pH 4 and pH 9, indicates that simultaneous determination of leflunomide with NSAIDs by conventional UV spectroscopy is not possible. Therefore, first derivative spectra of leflunomide and the above interacting drugs, having zero-crossing points at pH 4 (Figure 4a) and at pH 9 (Figure 4b) were used for their simultaneous determination without any prior separation in mixtures.

The absorbance at zero crossing points of leflunomide and interacting NSAIDs were chosen for their simultaneous determination. By constructing standard calibration curves between first derivative values of these drugs versus their concentration, Beer's law was confirmed (Tables 5,6). Least-squares method was used to determine regression curves. The linear calibration regression function for the spectroscopic determination of analyte at selected wavelength is given by $y=mx+c$, where y, m, x represents the absorbance, slope of linear regression and concentration of analyte (mMole), respectively while c is the intercept value, which reflects the difference between ideal and the real system. Two equation sets were used for the measurement of binary mixtures at two selected wavelengths (Tables 5 and 6). The first derivative spectra showed the best linear response to analyte concentration used at these wavelengths.

After determining the concentration of leflunomide and interacting NSAIDs by their, respective equations (Tables 5 and 6), their percentage availability were calculated (Table 7). Data was analyzed by student t-test keeping $p<0.05$ significant and $p<0.005$ as highly significant.

On HPLC: The developed and validated RP-HPLC method was successfully applied to study *in vitro* interactions between leflunomide and above mentioned NSAIDs (Figures 5a and 5b). Concentration of each drug was determined using linear equation after every 30 minutes and percentage availability was calculated. Data was also analyzed by student t-test using $p<0.05$ and $p<0.005$ as significant and highly significant, respectively. These studies indicated that percentage availability of leflunomide with all mentioned NSAIDs became altered at pH 4 and pH 9 which can directly affect its efficacy. Similar situation was also faced by these mentioned NSAIDs. Student t-test indicated that interaction of leflunomide with flurbiprofen (Table 8) became highly significant after 120 minutes at pH 4. At pH 9, this interaction was not as prominent as in case of pH 4 where interaction turned to highly significant ($p<0.005$) after 150 minutes.

Similarly, student t-test also indicated that leflunomide interacts with ibuprofen at both pH in highly significant ($p<0.005$) manner after 150 minutes. Diclofenac sodium interacted with leflunomide significantly just within one hour of reaction at both pH. This interaction became highly significant ($p<0.005$) within 60 minutes at pH 4 but at pH 9, reaction took 150 minutes to become highly significant. The potential interaction of leflunomide and mefenamic acid was studied only at pH 9 because at pH 4 this drug is insoluble. At pH 9, student t-test indicated that during whole procedure the



Precision	Factors changed								
	pH \pm 0.05			Flow rate(ml. min ⁻¹) \pm 0.2			Methanol % \pm 0.2%		
	2.65	2.7	2.75	1.45	1.5	1.55	79.8	80	80.2
Lef %RSD	0.62	0.78	0.65	0.99	0.78	0.39	0.23	0.78	0.73
Flur %RSD	0.079	0.534	1.04	0.78	0.534	0.25	0.62	0.534	0.96
Ibu %RSD	0.58	0.166	0.35	0.056	0.166	0.87	1.02	0.166	1.03
Mef %RSD	0.742	0.016	1.05	0.044	0.016	1.08	0.029	0.016	0.57
Diclo %RSD	0.43	0.29	0.48	0.82	0.29	0.92	0.45	0.29	0.43

Lef=leflunomide, Flur=flurbiprofen, Ibu=ibuprofen, Mef=mefenamic acid, Diclo=diclofenac sodium. n=6

Table 4: %RSD during robustness analysis.

S. No	Drug	Wavelength (nm)	Linearity range (mMole)	Regression equation	Correlation coefficient
1	Lef	243.6	0.01-.055	Y=0.457x -6*10 ⁻⁵	0.9987
	Diclo	258.4	0.09-0.18	Y=0.244x -6*10 ⁻⁵	0.9986
2	Lef	246.1	0.01-0.055	Y=0.435x +0.0011	0.9987
	Flur	258.2	0.06-0.15	Y=0.442x -0.008	0.999
3	Lef	248.1	0.01-0.055	Y=0.430x -0.003	0.9993
	Ibu	231.4	0.01-0.08	Y=0.615x+-0.003	0.9926

Lef=leflunomide, Flur=flurbiprofen, Ibu=ibuprofen, Mef=mefenamic acid Diclo=diclofenac sodium

Table 5: Regression analysis of leflunomide and NSAIDs at pH 4 by first order derivative spectrophotometry.

S. No	Drug	Wavelength (nm)	Linearity range (mMole)	Regression equation	Correlation coefficient
1	Lef	243.6	0.01-.055	Y=0.429x - 4E-05	0.998
	Diclo	258.4	0.09-0.18	Y=0.294x - 0.001	0.984
2	Lef	246.1	0.01-0.055	Y= 0.449x + 8E-05	0.995
	Flur	258.2	0.06-0.15	Y=0.440x - 0.002	0.984
3	Lef	248.1	0.01-0.055	Y=0.488x - 0.000	0.983
	Ibu	231.4	0.01-0.08	Y=1.036x + 0.025	0.995
4	Lef	253.2	0.01-0.055	Y= 0.244x + 7E-06	0.997
	Mef	231.4	0.02-0.065	Y=1.561x - 0.023	0.999

Lef=leflunomide, Flur=flurbiprofen, Ibu=ibuprofen, Mef=mefenamic acid, Diclo=diclofenac sodium

Table 6: Regression analysis of leflunomide and NSAIDs at pH 9 by first order derivative spectrophotometry.

Time (min)	At pH 4 % Availability (mean \pm S.D)		At pH 9 % Availability(mean \pm S.D)	
	Lef	Flur	Lef	Flur
0	100.35 \pm 0.98	99.99 \pm 0.98	100.1 \pm 0.42	100.28 \pm 0.11
30	99.55 \pm 0.51	98.45 \pm 0.25	100.4 \pm 0.51	100.1 \pm 0.25
60*	99.79 \pm 0.26	105.47 \pm 0.50	100.3 \pm 0.26	99.89 \pm 0.51
90*	101.61 \pm 0.61	109.58 \pm 0.52	99.87 \pm 0.61	98.78 \pm 0.52
120*	98.00 \pm 1.02	109.43 \pm 0.40	99.99 \pm 0.99	99.15 \pm 0.45
150**	94.24 \pm 1.54	108.97 \pm 0.64	102.1 \pm 1.08	100.15 \pm 0.89
180***	100.55 \pm 0.31	107.41 \pm 1.01	103.75 \pm 0.51	101.87 \pm 0.49
	Lef	Ibu	Lef	Ibu
0	100.22 \pm 0.98	100.17 \pm 0.24	100.7 \pm 0.71	100.33 \pm 0.325
30	104.32 \pm 0.46	103.97 \pm 0.35	101.75 \pm 0.778	101.23 \pm 0.478
60	107.56 \pm 0.45	107.08 \pm 0.13	101.65 \pm 0.212	101.42 \pm 0.60
90*	108.54 \pm 0.41	114.33 \pm 0.91	101.2 \pm 1.23	101.39 \pm 0.55
120*	105.32 \pm 0.32	107.54 \pm 0.42	102.225 \pm 0.38	100.65 \pm 0.489
150***	105.92 \pm 0.11	108.29 \pm 0.53	101.95 \pm 0.49	99.93 \pm 0.98
180***	105.07 \pm 0.178	117.87 \pm 0.19	102.35 \pm 0.65	98.93 \pm 0.51
	Lef	Diclo	Lef	Diclo
0	100.7 \pm 0.707	100.3 \pm 0.21	101.04 \pm 0.58	100.09 \pm 0.98
30*	103.36 \pm 0.51	98.85 \pm 0.23	102.25 \pm 0.31	100.52 \pm 0.75
60***	110.41 \pm 0.58	98.79 \pm 0.29	103.25 \pm 0.22	100.84 \pm 0.23
90***	112.95 \pm 0.19	96.76 \pm 0.33	104.3 \pm 0.54	101.14 \pm 0.46
120***	111.19 \pm 0.268	96.77 \pm 0.403	106.6 \pm 0.19	100.84 \pm 0.15
150****	111.76 \pm 0.23	96.91 \pm 0.321	104.8 \pm 0.412	101.69 \pm 0.28
180****	108.05 \pm 0.71	97.94 \pm 0.46	105.86 \pm 0.313	101.83 \pm 0.42
	Lef		Lef	Mef
0	-	-	100.15 \pm 0.21	100.08 \pm 0.268
30	-	-	101.75 \pm 0.35	101.82 \pm 0.247

60	-	-	100.65 ± 0.49	101.79 ± 0.49
90	-	-	100.69 ± 0.43	100.69 ± 0.43
120	-	-	100.42 ± 0.82	101.39 ± 0.19
150	-	-	102.7 ± 0.65	101.73 ± 0.558
180*	-	-	102.7 ± 0.42	101.73 ± 0.38

Lef=leflunomide, Flur=flurbiprofen, Ibu=ibuprofen, Mef=mefenamic acid, Diclo=diclofenac sodium. * and ** indicates p< 0.05 (significant) and p< 0.005 (highly significant)at pH 4 while * and ** indicates p< 0.05 (significant) and p< 0.005 (highly significant) interaction at pH 9

Table 7: Interaction study of leflunomide with NSAIDs by proposed UV method.

Time (mins)	At pH 4 % Availability (mean ± S.D)		At pH 9 % Availability(mean ± S.D)	
	Lef	Flur	Lef	Flur
0	100.06 ± 0.21	99.99 ± 0.27	100.02 ± 0.35	100.06 ± 0.09
30	100.5 ± 0.42	101.01 ± 0.22	98.04 ± 0.61	107.97 ± 0.81
60*	100.72 ± .51	102.65 ± 0.13	102.4 ± 0.51	103.25 ± 0.57
90*	99.89 ± .13	105.8 ± 0.61	100.12 ± 0.43	99.99 ± 0.26
120**	98.33 ± .71	113.52 ± 0.99	102.14 ± 0.76	101.7 ± 0.49
150***	96.38 ± .19	117.55 ± 0.42	104.28 ± 0.27	102.58 ± 0.94
180****	93.38 ± 18	131.62 ± 0.51	99.86 ± 0.772	105.88 ± 0.21
	Lef	Ibu	Lef	Ibu
0	100.1 ± 0.98	100.01 ± 0.74	100.25 ± 0.98	99.97 ± 0.11
30	97.98 ± 0.510.29	98.15 ± 0.26	100.39 ± 0.57	98.04 ± 0.52
60	98.09 ± 0.34	98.68 ± 0.13	104.18 ± 0.64	102.75 ± 0.41
90*	99.031 ± 0.32	98.2 ± 0.27	104.09 ± 0.26	103.46 ± 0.49
120*	103.33 ± 0.23	99.3 ± 0.53	103.53 ± 0.94	102.91 ± 0.75
150****	101.35 ± 0.24	97.9 ± 0.49	105.89 ± 0.25	103.07 ± 0.64
180****	103.64 ± 0.19	96.6 ± 0.21	108.69 ± 0.16	104.74 ± 0.28
	Lef	Diclo	Lef	Diclo
0	100.1 ± 0.23	100.15 ± 0.74	100.02 ± 0.64	99.99 ± 0.51
30*	101.33 ± 0.54	104.9 ± 0.16	99.89 ± 0.91	99.29 ± 0.42
60***	102.16 ± 0.86	110.11 ± 0.98	99.29 ± 0.83	95.60 ± 0.26
90***	101.4 ± 0.64	111.85 ± 0.23	102.79 ± 0.43	105.78 ± 0.54
120***	107.25 ± 0.35	110.15 ± 0.34	99.89 ± 0.71	97.65 ± 0.19
150****	110.19 ± 0.46	125.2 ± 0.56	102.46 ± 0.43	99.04 ± 0.18
180****	112.12 ± 0.28	126.67 ± 0.47	105.23 ± 0.22	101.58 ± 0.29
			Lef	Mef
0	-	-	100.91 ± 0.23	100.04 ± 0.43
30	-	-	100.7 ± 0.52	100.65 ± 0.61
60	-	-	100.61 ± 0.16	100.01 ± 0.78
90	-	-	101.13 ± 0.14	101.27 ± 0.98
120	-	-	101.51 ± 0.09	100.27 ± 0.15
150	-	-	101.99 ± 0.21	102.05 ± 0.11
180*	-	-	102.78 ± 0.84	101.99 ± 0.31

Lef=leflunomide, Flur=flurbiprofen, Ibu=ibuprofen, Mef=mefenamic acid, Diclo=diclofenac sodium. * and ** indicates p< 0.05 (significant) and p< 0.005 (highly significant)at pH 4 while * and ** indicates p< 0.05 (significant) and p< 0.005 (highly significant) interaction at pH 9

Table 8: Interaction study of leflunomide with NSAIDs by proposed RP- HPLC method.

interaction was insignificant (p>0.05) except at 180 minutes (p=0.023) which was significant (p<0.05) (Table 8).

Conclusion

A facile reversed-phase HPLC-UV method for the simultaneous determination of leflunomide, flurbiprofen, ibuprofen, diclofenac sodium and mefenamic acid been developed for the first time. In addition to its novelty for simultaneously determining four NSAIDs with leflunomide in two different phases and using two different columns the method is sufficiently rapid, simple, sensitive as well as precise and accurate that complies with ICH guidelines [19] for accuracy, precision and stability for standards and QC samples. The detector response was found to be linear over a wide concentration range having LLOD for flurbiprofen, ibuprofen, diclofenac sodium and mefenamic acid 6.9, 296, 71 and 12 µg mL⁻¹ respectively. No interference of extra pharmaceutical ingredients was observed in the assay and was

successfully applied in raw materials and pharmaceutical formulations. The main advantage of the proposed analytical method is the use of the same mobile phase for the determination leflunomide and NSAIDs in bulk and in dosages formulations. Hence, it can be recommended for the routine quality control of these drugs. In addition to the analysis of these drugs, this rapid and reproducible analytical method is suitable for dissolution studies and can also be used for routine clinical, pharmacokinetic and interaction studies conducted in humans as they are prescribed simultaneously to patients of rheumatoid arthritis.

The developed method has been applied to study *in vitro* interactions between leflunomide and flurbiprofen, ibuprofen, diclofenac sodium and mefenamic acid in simulated human body environments. The results of interaction studies showed that activity of leflunomide may be affected in presence of NSAIDs when given simultaneously. In this *in vitro* studies, it is observed that flurbiprofen, ibuprofen and diclofenac sodium affect leflunomide's availability in a

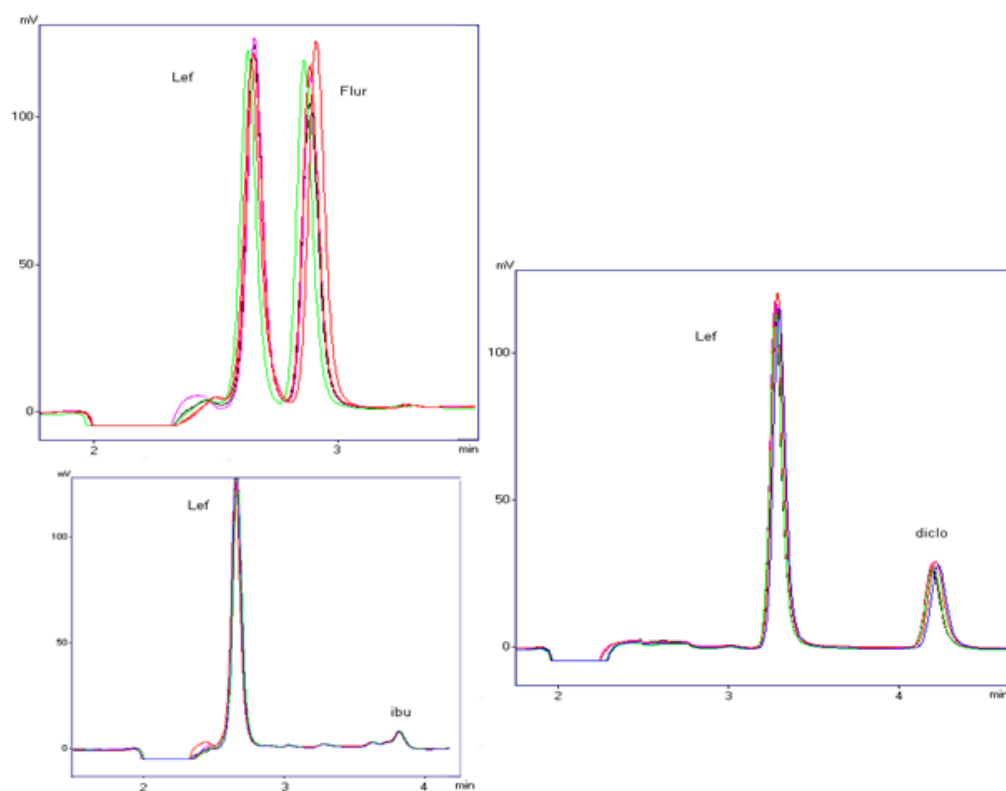


Figure 5a: Drug interacting chromatograms of leflunomide and NSAIDs at pH 4.

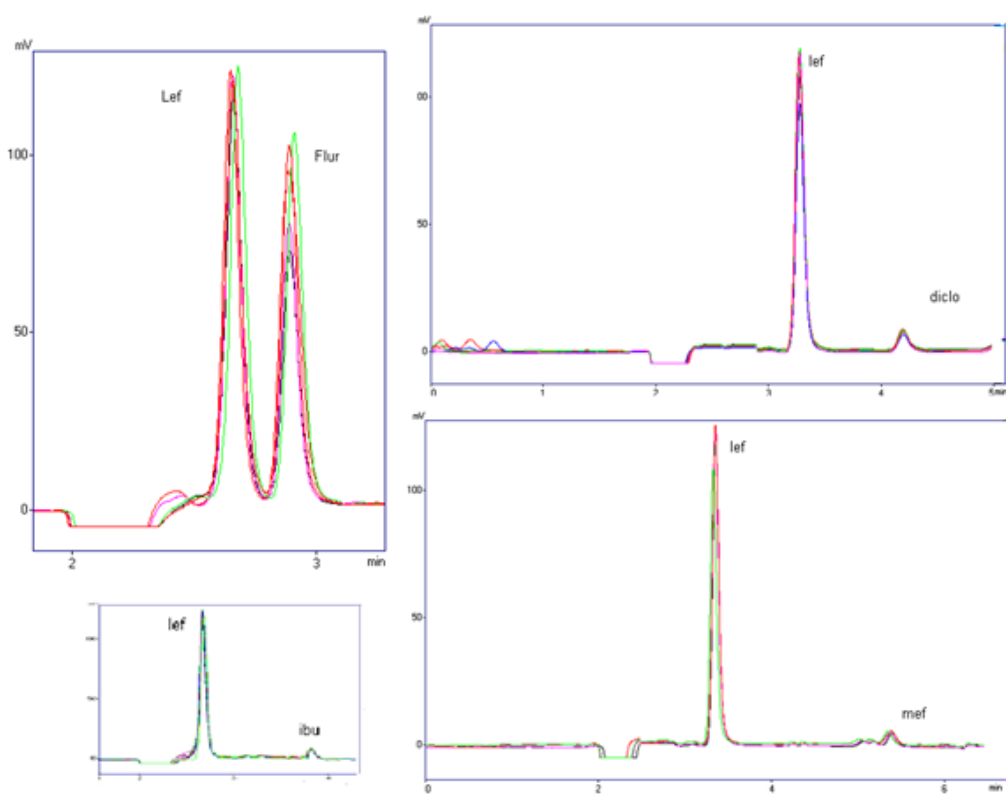


Figure 5b: Drug interacting chromatograms of leflunomide and NSAIDs at pH 9.

highly significant ($p < 0.005$) manner as compared to mefenamic acid however there *in vivo* detailed interaction should be studied in future.

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