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Full Length Research Paper

Development and validation of LC method for the determination of leflunomide in pharmaceutical formulations using an experimental design

Srinivas Rao V[2], Sunanda K. K[3], Narasimha Rao M.[1], Allam Appa Rao[1], Maheswari IL[1] and Srinubabu G[1]*

[1]Center for Biotechnology, Andhra University College of Engineering (A), Visakhapatnam- 530003, India.
[2]St John's Pharmacy College, Vijayanagar, Bangalore-40, India.
[3]Department of Microbiology, Andhra University College of Science and Technology, Visakhapatnam-530003, India.

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A rapid and sensitive RP-HPLC method with UV detection (260 nm) for routine analysis of leflunomide in a pharmaceutical formulation was developed. Chromatography was performed with mobile phase containing a mixture of acetonitrile and phosphate buffer (60:40, *v/v*) with flow rate of 0.8 ml min^{-1}. Quantitation was accomplished with internal standard method. The procedure was validated for linearity (correlation coefficient = 0.9999), accuracy, robustness and intermediate precision. Experimental design was used for validation of robustness and intermediate precision. Plackett-Burman design was used to screen the essential factors for optimization. To test robustness, three factors were considered; percentage v/v of acetonitrile in mobile phase, flow rate and pH; an increase in the flow rate results in a decrease of the drug found concentration, while the percentage of organic modifier and pH have no important effect on the response. For intermediate precision measure the variables considered were: analyst, equipment and number of days. The RSD value (0.93%, *n* = 24) indicated a good precision of the analytical method. The proposed method was simple; highly sensitive, precise, accurate and retention time less than 6 min indicating that the method is useful for routine quality control.

Key words: Leflunomide, HPLC, validation, Robustness testing, experimental design.

INTRODUCTION

The chemical name for leflunomide is N- (4´-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide. It has a molecular weight of 270.2 and has structural formula of (Figure 1) which is an isoxazole derivative and inhibitor of de novo pyrimidine synthesis, (Katarzyna et al., 1998; Migita et al., 2005) represents a new class of disease modifying anti rheumatic drugs. The primary mode of action of leflunomide is specific inhibition of dihydro-orotate dehydrogenase, a key enzyme in the de novo synthesis of pyrimidine, and subsequent inhibition of RNA and DNA synthesis (Fox, 1998) Activated T lymphocytes, which predominantly synthesize pyrimidines via the de novo pathway (Fairbanks et al., 1995), may be especially susceptible to leflunomide. The immunomodulatory and anti-inflammatory effects of leflunomide have recently been reviewed by Madison et al. (1999) and include blockade of tumor necrosis factor mediated activation of the transcription factor NFêB (Manna and Aggarwal, 1999); inhibition of reactive oxygen radicals (Krann et al.,2000);inhibition of polymerphonuclear leucocyte chemotaxis and the migration of polymorphonuclear leucocytes into the rheumatoid synovial cavity (Deage, et al., 1998) and inhibition of matrix metalloproteinases and subsequent increases in tissue inhibitor of metalloproteinase (TIMP)/MMP ratios *in vitro*[9] and in patients with RA (Krann et al, 2000) its metabolism as discussed by Amit et al. (2003). Several methods were reported for the determination of leflunomide in plasma (Fairbanks et al., 1999; Lusien et al., 1995; Schmidt et al., 2003; Chan et al., 2004; Roon et al., 2004). A more recent paper describing the pharmaceutical determination describing the pharmaceutical deter-

*Corresponding author. Email: srinubabuau6@gmail.com. Phone: +91-891-2844204

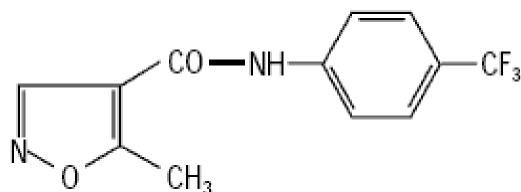

Figure 1. Structure of leflunomide.

determination of leflunomide by FIA-UV (Yeniceli et al., 2005) has been has been reported. Recently Yeniceli et al. (2006) reported its determination in tablet dosage forms. Besides costly instrumentation all these methods needs gradient elution and more time takes to analysis. This paper reports a rapid and sensitive HPLC determination method with UV detection, useful for routine quality control of leflunomide in pharmaceutical formulations. The method was validated by parameters such as linearity, accuracy, precision and robustness. Experimental design was used for validation to evaluate the robustness and intermediate precision.

Experimental

Apparatus

Two different HPLC systems were used for the study. The corresponding specifications were provided below.

HPLC system 1: The HPLC 1 apparatus was a waters chromatographic system equipped with an injection valve (Rheodyne 033381); Waters 2487 UV dual λ absorbance detector was used. A reversed-phase C_{18} column (25 cm × 4.6 mm i.d., particle size 5 µm). Peak area integration was performed using Breeze software.

HPLC system 2: The HPLC 2 apparatus was a Shimadzu chromatographic system with two LC-10AT VP pumps, variable wavelength programmable UV/Visible detector SPD-10A, VP CTO, -10 AS VP column oven (Shimadzu) A reversed-phase C_{18} column (25 cm × 4.6 mm i.d., particle size 5 µm; YMC, IMC, Wilmington, NC, 28403, U.S.A.) and the HPLC system was monitored by software "Class-VP series version 5.03 (Shimadzu)". A model SL-164 UV-Visible spectrophotometer (Elico Ltd, India) was employed for spectrophotometric study.

The experimental design and statistical analysis of the data were performed, by statistica (Stat soft, 2001) data analysis software system, by using Factorial design (9 runs) for robustness study.

Reagents

HPLC grade acetonitrile, potassium dihydrogen phosphate and orthophosphoric acid (A.R.grade) was obtained from Quiligens (Mumbai, India). Pure sample of drug and internal standard were obtained from Sun pharmaceuticals Ltd. Baroda India. Ultra pure water obtained using a Milli-Q® UF-Plus apparatus (Millipore) and the same was used to prepare all solutions for the method. The determination of leflunomide in commercial formulation was carried out using (lefra®) 20 mg tablets. The pH of the phosphate buffer maintained at ≈ 3.0

Preparation of the standard solution

An accurately weighed sample (100 mg) of leflunomide reference standard was transferred to a 100 ml volumetric flask and dissolved in triple distilled water to make a solution (1 mg ml^{-1}). From this solution, a working standard solution of 100 µg $ml^{-1}$of strength was prepared from this dilution of 1,5, 10, 20, 30, and 40 µg ml^{-1} were made in 10 ml volumetric flasks with the mobile phase here in every standard solution contains 5 µg ml^{-1} of pramipexole internal standard. To carry out the assay aliquots of leflunomide solution equal to 1, 5, 10, 20, 30 and 40µg ml^{-1} were accurately withdrawn. 20µl of each sample was injected into the system.

Extraction of leflunomide from tablets

About 20 tablets of lefra® (each tablet contains 20 mg of leflunomide as API) were weighed and thoroughly powdered. The amount of powder equivalent to about 20 mg was placed in a 100 ml volumetric flask. To it around 90 ml of solvent (methanol) was added and the flask was placed in an ultrasonic bath for 10 min and then the solution was centrifuged at 3000 rpm for 15 min. The supernatant was diluted to volume with the same solvent. The solution was filtered though a 0.45 µm filter and then the filtrate were used to prepare sample solutions of different concentrations

Calibration procedure

The calibration curve was plotted with six concentrations of the standard solutions 1 – 40 µg ml^{-1} and chromatography was repeated thrice for each dilution. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method. Before injecting solutions, the column was equilibrated for at least 30 min. with the mobile phase flowing through the system. Quantitation was accomplished using an internal standard method. Five determinations were carried out for each solution. Peak area ratios were recorded for all the solutions. The correlation graph was constructed by plotting the peak area ratios obtained at the optimum wavelength of detection versus the injected amounts.

Chromatographic conditions

The mobile phase was a mixture of Acetonitrile and phosphate buffer (60:40, *v/v*) and flow rate was 0.8 mL min^{-1}. The UV detector wavelength was set at 260 nm and the temperature was set at ≈ 23°C.

RESULTS AND DISCUSSION

The applied chromatographic conditions permitted a good separation of leflunomide 10 µg ml^{-1} and the internal standard pramipexole 10 µgml^{-1} (Figure 2), no drug decomposition was observed during the analysis. The LC method was validated for the parameters reported below.

System suitability

The chromatographic separation, as explained above was carried out with HPLC 1 to evaluate the chromatographic parameters (capacity factor (K′), asymmetry of the peaks, tailing factor and resolution between two con-

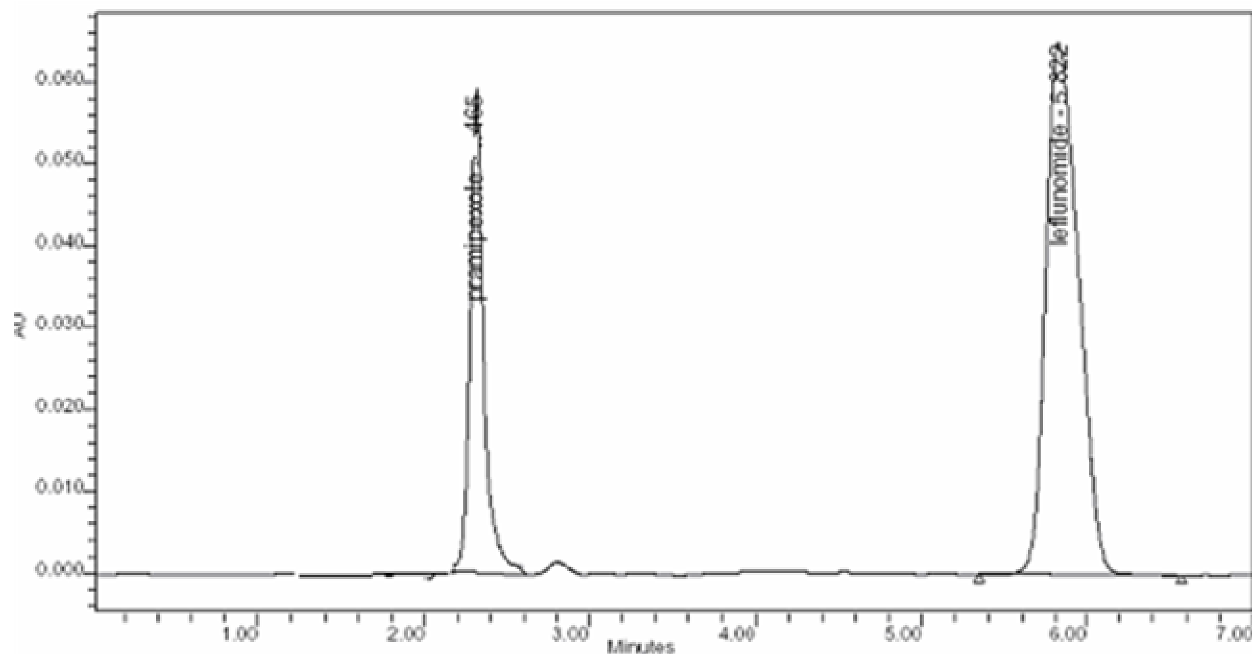

Figure 2. Chromatogram of leflunomide with IS pramipexole at described conditions.

Table 1. Results of the data analysis for the quantitative determination of leflunomide by the proposed method.

Statistical parameter	HPLC
Concentration range (µg/ml)	1-40
Regression equation	y = 0.31959x-0.08903
Correlation coefficient (r)	0.9999
Stand error on estimation(S_e)	0.09693
Standard deviation on slope(S_b)	0.00235
Standard deviation on intercept(S_a)	0.05383
Limit of detection (LOD) (µg/ml)	0.50
Limit of quantification (LOQ) (µg/ml)	2

secutive peaks) In Figure 2 representative chromatogram was shown, which corresponds to the chromatographic separation of these substances. The capacity factor (K′) of the first peak was 2.6 and second was 7.3, the resolution factor was 2.6, results obtained for asymmetry of the peak and tailing factor parameters were the following 0.781 and 0.645 respectively for IS pramipexole, 0.324 and 0.432 for leflunomide respectively.

It was concluded that the developed method is the optimum according to the studied parameters. The capacity factor obtained is within the accepted values, above 2 for the first peak and less than 10 for the second peak. The tailing factor to be controlled was within the limits established by these guidelines. Lastly, good resolution was obtained between two consecutive peaks in the developed method. Therefore this method can be applied to its intended purpose with no problems, its suitability being proved.

Stability of the solution

Results obtained in the study of the solution (both reference and sample solution) where It can be noticed that solutions were stable for 48 h, as during this time the results does not decrease below the minimum percenttage (98%).

Linearity

Leflunomide and internal standard were chromatographed using the mobile phase. The linearity of peak area responses versus concentrations was studied from 1 - 40 µg ml^{-1} for leflunomide. A linear response was observed over the examined concentration range. The re-

Table 2. Accuracy for leflunomide.

Concentration (µg ml^{-1})	Recovery (%)[a]	RSD (%)
5	96.85	1.25
10	102.28	1.65
20	97.74	1.08
Mean	98.92	1.33

[a]mean of five determinations.

Table 3. Chromatographic conditions and range investigated during robustness testing.

Variable	Optimized value	Range investigated
Mobile phase (ACN/buffer	60:40	50 -70
Flow rate (ml min^{-1})	0.8	0.6 - 1.0
pH	3.0	2.5 – 3.5

Table 4. Experimental domain for Plackett-Burman design.

%ACN in Mobile phase	Flow rate	Auto sampler temp	Column oven temp	Detector wavelength	p^H	Injection volume	
85	0.2	5	25	30	25	8	-1
95	0.4	15	35	40	30	12	1

results are tabulated in Table 1.

Accuracy and repeatability

Accuracy was studied using three different solutions, containing 5, 10 and 20 µg ml^{-1} of leflunomide. Recovery data are reported in Table 2. The obtained values were within the range of 96.85 and 102.30%, mean RSD% was 1.33%, satisfying the acceptance criteria for the study. The system repeatability was calculated from five replicate injections of leflunomide at the analytical concentration of about 10 µg ml^{-1}; the RSD% found was 0.82.

Robustness

As defined by the ICH, the robustness of an analytical procedure refers to its capability to remain unaffected by small and deliberate variations in method parameters (ICH, 1997). In order to study the simultaneous variation of the factors on the considered responses, a multivariate approach using design of experiments is recommended in robustness testing. A response surface method was carried out to obtain more information and to investigate the behavior of the response around the nominal values of the factors. Response surface methodology (RSM) has the following advantages: (a) to allow a complete study where all interaction effects are estimated; and (b) to give an accurate description of an experimental region around a center of interest with validity of interpolation (Myers and Montgomery, 1995). Generally the large numbers of experiments required by standard designs applied in RSM discourage their use in the validation procedure. In this study two level Plackett-Burman design was employed at two levels (-1, +1) for evaluation of essential factors from % acetonitrile in mobile phase (%ACN), flow rate (FR), auto sampler temperature (AST), column oven temperature (COT), detector wavelength (DW), pH, injection volume (IV) (all are qualitative factors) as shown in Table 6. The ANOVA results (Table 6) such as p-values 0.05234 for flow rate, 0.0907 for % acetonitrile and 0.0571 for p^H (Table 4, 5) indicated that these factors are important than other factors indicated above. The R^2 = 0.9983 indicated that the model was fit for the applied experimental design, further optimization should have to perform to report critical values.

However, if an analytical method is fast and requires the testing of a few factors (three or less), a good choice for robustness testing may be the factorial design (Yates and Kenneth, 1963) widely employed because of its high efficiency with respect to the number of runs required. In factorial design k factors requires 3^k factorial runs, symmetrically spaced at $\pm\alpha$ along each variable axis, and at least one center point. In order to study the variables at no more than three levels (–1, 0, +1), the design used in robustness testing of leflunomide was a factorial design with α = ±1 (Montgomery, 2003). Three factors were

Table 5. Experimental plan for Plackett – Burman design and obtained responses.

%ACN in mobile phase	low rate	Auto sampler temp	Column oven temp	Detector wavelength	Inj.vol.	pH	Peak Area ratio
-1	-1	1	1	-1	-1	1	2.76
1	-1	1	-1	1	-1	-1	2.74
-1	1	-1	-1	1	-1	1	2.84
-1	1	1	-1	-1	1	-1	2.73
1	-1	-1	-1	-1	1	1	2.78
1	1	-1	1	-1	-1	-1	2.82
1	1	1	1	1	1	1	2.91
-1	-1	-1	1	1	1	-1	2.79
1	1	1	1	1	1	1	2.92

Table 6. ANOVA results for Plackett – Burman design.

SS	MS	F	p	Factor
0.002430	0.002430	48.6000	0.090700	%ACN
0.007363	0.007363	147.2667	0.052342	FR
0.000963	0.000963	19.2667	0.142602	AST
0.005070	0.005070	101.4000	0.063014	COT
0.005070	0.005070	101.4000	0.063014	DW
0.000403	0.000403	8.0667	0.215518	pH
0.006163	0.006163	123.2667	0.057186	I V

Table 7. Experimental plan for robustness testing and obtained responses

No. exp	Acetonitrile (%)	Flow rate (ml min^{-1})	pH	Peak area ratio
1	70	0.6	3.0	2.93
2	70	1.0	3.5	2.84
3	70	0.8	2.5	2.91
4	60	0.8	3.0	2.95
5	60	0.6	3.5	2.92
6	50	1.0	3.0	2.82
7	50	0.8	3.5	2.89
8	55	1.2	2.5	2.86
9	50	0.6	2.5	2.89

considered: percentage v/v of acetonitrile (x_1); flow rate ml min^{-1} (x_2) and pH (x_3). The ranges examined were small deviations from the method settings and the corresponding responses in the peak area ratio considered (*Y*) were observed. A factorial design with 9 experiments, including the center point. The experimental plan and the corresponding responses are reported in Table 7, all experiments were performed in randomized order to minimize the effects of uncontrolled factors that may introduce a bias on the response. A classical second-degree model with a cubic experimental domain was postulated. Experimental results were computed by statistica (Stat soft, 2001). The coefficients of the second-order polynomial model were estimated by the least squares regression. The equation model for *Y* (found peak area ratio) was as follows:

$$Y= 2.891111+0.011667x_1-0.038333x_2-0.003333x_3-0.014167x_1^2+0.019167x_2^2 -0.00666x_3^2$$

The factor flow rate (x_2) was significant for the regression model assumed. The model was validated by the analysis of variance (ANOVA). The statistical analysis showed (Table 8) that the model represents the phenomenon quite well and the variation of the response was correctly

Table 8. ANOVA results.

Parameter	SS	MS	F	P
ACN (%)	0.00242	0.00121	8.384	0.106
Flow rate (ml/min	0.01175	0.00587	40.692	0.020
Ph	0.0004	0.0002	1.461	0.406

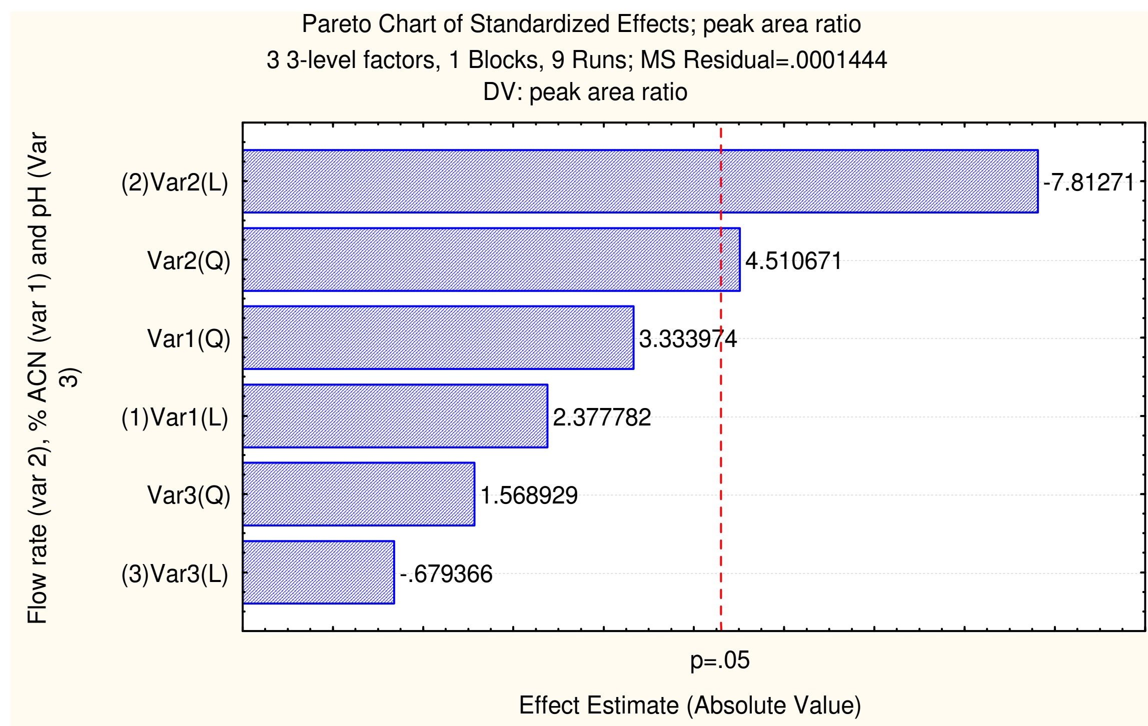

Figure 3. Representative graph to show the influence of variables studied in the response of leflunomide.

related to the variation of the factors, Figure 3 shows the influence of each of the variables studied in the leflunomide as a response where none of them exceeds the limit except the flow rate as shown in the pareto graph. The interpretation of the results has to start from the analysis of the whole model equation rather than from the analysis of the single coefficients. It is important for the response surface study, to consider also the factors whose coefficients are statistically non-significant. For this reason the analysis of the response surface plot is necessary. As shown in Figure 4(a–c), the analysis produces three-dimensional graphs by plotting the response model against two of the factors, while the third is held constant at a specified level, usually the proposed optimum. Figure 3 shows a graphical representation of the isoresponse surface for variation of percentage of ACN (x_1) and flow rate (x_2), while the p^H (x_3) is maintained constant at its optimum of 3.0. An increase in the flow rate results in a decrease of the observed peak area ratio (Y), while the percentage of organic modifier had no important effect on the response. Analogous interpretation may be derived by examining Figure 4c that plots the factors flow rate (x_2) versus pH (x_3). In Figure 4b, where the factor flow rate is maintained constant, the method can be considered robust for the studied experimental response. In conclusion, by examining the ANOVA results and analysis of response surface confirms that Y is not robust for factor x_2, thus a precautionary statement should be included in the analytical procedure for this factor.

Intermediate precision

The intermediate precision is a measure of precision between repeatability and reproducibility and it should be established according to the circumstances under which the procedure is intended to be used (ICH, 1997). The analyst should establish the effects of random events on the precision of the analytical procedure. The intermediate precision is obtained when the assay is performed by multiple analysts, using multiple instruments, on multiple days, in one laboratory (ICH, 1997). In order to study

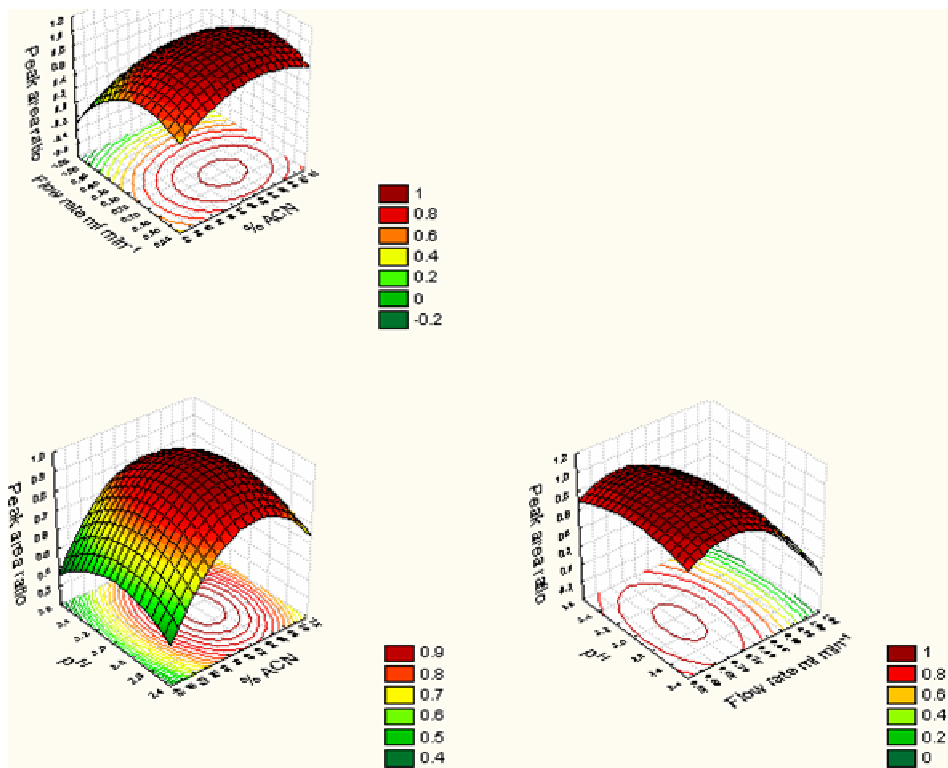

Figure 4. Three-dimensional plot of the response surface for *Y* (found drug peak area ratio). (a) Variation of the response *Y* as a function of x_1 (% Acetonitrile) and x_2 (flow rate); fixed factor: x_3 (p^H) = 3.0 (c) Variation of the response *Y* as a function of x_2 (flow rate) and x_3 (p^H); fixed factor: x_1 (% Acetonitrile)=60% *v/v*. (b) Variation of the response *Y* as a function of x_1 (% Acetonitrile) and x_3 (p^H) fixed factor: x_2 (flow rate) = 0.8 ml min^{-1}

Table 9. Exprimental plan for intermediate precision testing and obtained responses

No. exp.	Analyst	Instrument	Day	peak area ratio
1	Analyst 1	HPLC 1	Day 1	2.89
2	Analyst 1	HPLC 1	Day 1	2.85
3	Analyst 1	HPLC 1	Day 1	2.84
4	Analyst 2	HPLC 1	Day 1	2.83
5	Analyst 2	HPLC 1	Day 1	2.84
6	Analyst 2	HPLC 1	Day 1	2.81
7	Analyst 1	HPLC 2	Day 1	2.92
8	Analyst 1	HPLC 2	Day 1	2.93
9	Analyst 1	HPLC 2	Day 1	2.94
10	Analyst 2	HPLC 2	Day 1	2.91
11	Analyst 2	HPLC 2	Day 1	2.96
12	Analyst 2	HPLC 2	Day 1	2.94
13	Analyst 1	HPLC 1	Day 2	2.80
14	Analyst 1	HPLC 1	Day 2	2.81
15	Analyst 1	HPLC 1	Day 2	2.80
16	Analyst 2	HPLC 1	Day 2	2.80
17	Analyst 2	HPLC 1	Day 2	2.86
18	Analyst 2	HPLC 1	Day 2	2.91
19	Analyst 1	HPLC 2	Day 2	2.92
20	Analyst 1	HPLC 2	Day 2	2.90
21	Analyst 1	HPLC 2	Day 2	2.89
22	Analyst 2	HPLC 2	Day 2	2.93
23	Analyst 2	HPLC 2	Day 2	2.90
24	Analyst 2	HPLC 2	Day 2	2.89

these effects simultaneously, a multivariate approach was used. The considered variables included analysts (1 and 2), equipment (HPLC 1 and 2) and days (1 and 2). The considered response was the found drug peak area ratio. A linear model ($y=b_0+b_1x_1+b_2x_2+b_3x_3$) was postulated and a 2^3 full factorial design was employed to estimate the model coefficients (Srinubabu et al., 2006, 2007). Each experiment was repeated three times in order to evaluate the experimental error variance. The analyses were carried out in a randomized order according to the experimental plan reported in Table 9. The concentration of leflunomide was about 10 µg ml^{-1}. No considered factor was found significant for the regression model assumed. The RSD found (0.93%, n = 24) was acceptable, indicating a good precision of the analytical procedure.

Conclusion

The proposed high-performance liquid chromatographic method has been evaluated over the linearity, precision, accuracy, and suitability and proved to be convenient and effective for the quality control of leflunomide in pharmaceutical dosage forms. The measured signal was shown to be precise, accurate, and linear over the concentration range tested (1.0 – 40.0 µg ml^{-1}) with a correlation coefficient 0.9999. Moreover, the lower solvent consumption along with the short analytical run time of 6.0 min leads to a cost effective and environmentally friendly chromatographic procedure. Thus the proposed methodology is rapid, selective, requires a simple sample preparation procedure, and represents a good procedure of leflunomide in tablets.

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