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Journal of Pharmaceutical and Biomedical Analysis 40 (2006) 197–201
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Determination of leflunomide in tablets by high performance liquid chromatography

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Received 24 January 2005; received in revised form 24 June 2005; accepted 28 June 2005
Available online 19 August 2005

Abstract

In the present study, a reverse phase high performance liquid chromatography (HPLC) method was validated and applied for the determination of leflunomide in tablets. Chromatographic separation of leflunomide and oxazepam as an internal standard was carried out on a C_{18} column (50 mm, 3 mm i.d.) using a mobile phase, consisting of methanol and water (60:40, v/v), at a flow rate of 0.5 ml min^{-1} and UV detection at 260 nm. The retention times for oxazepam and leflunomide were 2.6 and 5.2 min, respectively. The validated quantification range of the method was 2.7×10^{-6} to 5.5×10^{-5} M for leflunomide. The results of the developed procedure in tablets were compared with those of UV spectrophotometry to assess active leflunomide content.

Keywords: Leflunomide; HPLC; Tablet analysis; Pharmaceuticals

1. Introduction

Leflunomide (LEF), [*N*-(4′-trifluoromethylphenyl)-5-methyl-isoxazole-4-carboxamide] is a novel isoxazol derivative with both anti-inflammatory and immunosuppressive properties. The chemical structure of LEF is given in Fig. 1. It has been used to reduce the signs and symptoms of arthritis and to retard joint damage in patients with active rheumatoid arthritis. LEF is a prodrug, which is rapidly and non-enzymatically converted to its active metabolite, A77 1726 after oral administration. It is reported that A77 1726 possesses immunomodulator effects of the drug by reversible inhibition of the enzyme dihydroorotate dehydrogenase and inhibits cell proliferation of lymphocytes [1–4].

Since the conversion of LEF to A77 1726 in vivo is essentially complete, most pharmakokinetic studies have been focused to measure A77 1726. Several high performance liquid chromatography (HPLC) methods have been published for the kinetic monitoring and determination of A77 1726 in human blood and plasma [5–10]. It is also reported that the levels of LEF and A77 1726 in the cells and in the incubation media by LC–MS/MS and HPLC methods [11]. Among HPLC methods proposed up to date, reversed phase columns with different size have been used for the quantification of the major metabolite. In a simultaneous determination for LEF and A77 1726 in human plasma performed by HPLC, the retention time of LEF was reported about 16 min and also proposed for LEF containing pharmaceuticals [8]. It is relatively long time for an active content assay of LEF to use in routine laboratories. Recently, a pharmaceutical determination of LEF by FIA–UV detection has been reported [12].

This study describes a rapid, sensitive, accurate and precise method for the determination of LEF in tablets using HPLC. The method has been validated with respect to precision of peak response, linearity range, specificity and accuracy, limit of detection (LOD) and limit of quantification (LOQ). The proposed method has been applied to the analysis of LEF tablets, the results are compared to those obtained from UV spectrophotometry and statistically evaluated.

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doi:10.1016/j.jpba.2005.06.030

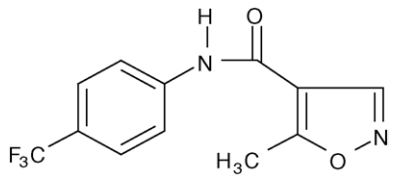

Fig. 1. The chemical structure of LEF.

2. Experimental

2.1. Reagents and chemicals

The standard LEF was obtained from Sigma (St. Louis, MO, USA). Methanol (gradient grade), hydrochloric acid, sodium hydroxide and hydrogen peroxide (30%) and tablet excipients (hydroxypropyl methyl cellulose, lactose monohydrate, magnesium stearate, polyethylene glycol 4000, povidone, maize starch, talc and titanium dioxide) were the products of Merck Co. (Darmstat, G) and they were all analytical-reagent grade. Therefore, all of them were used without any further purification. Double distilled water and ethanol used for the preparation of the solutions and they were prepared in all pyrex glass apparatus in our laboratory. The pharmaceutical dosage form containing 20 mg LEF, Arava®, a product of Aventis Pharma A.S. (Istanbul, Turkey) was purchased from a local drugstore. Oxazepam which was employed as an internal standard (IS) was obtained from Wyeth Ilaçları, A.S. (Istanbul, Turkey).

2.2. Standard preparation

A stock solution of LEF was prepared at a concentration of 1 mg ml^{-1} (10 ml) in ethanol [8], and serially diluted with aqueous ethanol solution (25%, v/v) to give working standard solutions in the range of 2.7×10^{-6} to 5.5×10^{-5} M. An IS solution of oxazepam (1 mg ml^{-1}) was also prepared in ethanol and used always at a fixed concentration of 1.4×10^{-5} M. Stock solutions and standards were stored in glass vials and they were covered with aluminum folia and kept at 4 °C.

2.3. Instrumentation

The HPLC system comprised of a model of LC-10A pump equipped with a manual injector, and a model of SPD-M10A diode array detector and the system was processed with Class-LC10 software controlling by CBM-10A communication module all Shimadzu (Kyoto, Japan). Standard solutions and samples were injected to a Rheodyne model by a 5 μl loop injection port (Cotati, CA, USA) with a 22-gauge injection needle. A model of UV-2401 PC spectrophotometer from Shimadzu for common spectrophotometric studies and B-220 sonicator from Branson (Danbury, CT, USA) for sonication were employed.

2.4. HPLC

An isocratic mobile phase consisting of methanol–water (60:40, v/v) was prepared, degassed and filtered from 0.45-μm membrane filter under a negative pressure before passing through the instrument. The flow rate was 0.5 ml min^{-1} and the detector was set to 260 nm to detect the signals. Chromatographic separation was performed on a Phenomenex (Torrance, CA, USA) Luna C_{18} column (3 μm spherical particle, pore diameter 100 Å, 3.0 mm i.d. × 50 mm). Always 5 μl standard and sample was injected to the column.

2.5. Assay validation

The method was validated according to ICH guidelines for validation of analytical procedures [13].

Calibration curves were obtained with six concentrations of the standard solutions ($n = 3$) in the range of 2.7×10^{-6} to 5.5×10^{-5} M. Linearity was evaluated by linear regression analysis using the least square regression method.

The precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was evaluated by assaying the samples of the same concentration during the same day. The intermediate precision was studied by comparing the assays on different days (3 days).

Accuracy of the analytical method was determined by analyzing both quality control samples prepared using standard LEF solution and synthetic inactive ingredients (matrix) solution by spiking with different known concentrations of LEF (within the calibration range). The matrix solution was prepared in ethanol using common tablet excipients such as hydroxypropyl methyl cellulose (7%), lactose monohydrate (60%), magnesium stearate (1%), polyethylene glycol 4000 (5%), povidone (5%), maize starch (5%), talc (1%), titanium dioxide (1%). Working standards of LEF were prepared at three concentration levels ($n = 6$ for each concentration) in an aqueous solution of ethanol (25%, v/v) and in the matrix solution. Percentage recoveries, percentage error and percentage R.S.D. values were used to express accuracy.

Specificity of the method was determined with stressed LEF solutions (2.0×10^{-4} M) after preparation in 0.1 N HCl, 0.1 N NaOH and 3% H_2O_2 and a treatment at room temperature and 60 °C with different time points such as 15, 30, 45, 60, 90, and 120 min. The samples for specificity test were injected to HPLC after tenfold dilution with a mobile phase.

System suitability for the proposed method was evaluated by using LC10A software. Repeatability for injection was also assessed by injecting six sample solutions (5.5×10^{-6} M). Data from replicate injection at this assay concentration was processed for calculations keeping 2% R.S.D. as limit. The parameters tested for system suitability included capacity factor, resolution, tailing, theoretical plates, retention time and percentage R.S.D. of injection repeatability.

Quantification of peaks was achieved by the ratio of peak area normalization values of LEF and IS calculated as [(peak area of LEF/retention time of LEF)/(peak area of IS/retention time of IS)].

2.6. Sample preparation

Ten Arava® tablets (each containing 20 mg LEF) were weighed and finely powdered in a mortar. The net weight of each tablet was calculated. A sufficient amount of tablet equivalent to the average weight of tablet content was accurately weighed and 10 ml ethanol was added to dissolve the active material ($n = 6$). It was sonicated for 10 min and then the solution was centrifuged at 5000 rpm for 10 min. The supernatant was diluted to obtain the concentrations would be in the available range of calibration studies

The same tablet solutions ($n = 6$) were used for the UV spectrophotometric determination of LEF. The necessary dilutions were made from that stock solution in the concentration range of 1.1×10^{-5} to 3.3×10^{-5} M and the measurements were performed at the wavelength of 260 nm by using 25% (v/v) ethanol as a blank.

3. Results and discussion

In this study, the chromatography of LEF was investigated on a short (50 mm) C_{18} 3.0 mm i.d. column because of the known advantages such as less solvent usage and short analysis time resulting in less band broadening and peak asymmetry [14,15]. LEF and oxazepam were separated by this column using mobile phase of methanol:water (60:40, v/v). Oxazepam was used as an IS to compensate for minor fluctuations of retention times. Retention times were 2.6 min for IS and 5.2 min for LEF as seen in Fig. 2.

Certain characteristics have been obtained from the chromatograms. These results from system suitability are presented in Table 1. Good agreement was found when the results were compared with the recommended values.

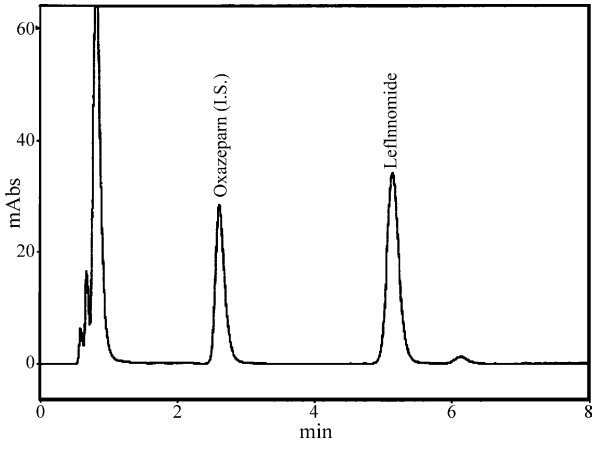

Fig. 2. Representative chromatograms of LEF (1.1×10^{-6} M) and IS (1.4×10^{-5} M) by the proposed method.

Table 1
System suitability parameters

Parameters	Observed value	Recommended value
Retention time (min)	5.2	
Capacity factor (k')	7.90	>2
Tailing factor (T)	1.20	≤2
Resolution (Rs)	8.61	>2
Theoretical plates (N)	3826	>2000
R.S.D.% (for Ret. time)	0.88	≤1

3.1. Method validation

3.1.1. Precision

Precision of the method was determined by repeatability (intra-day) and intermediate precision (inter-day) and was expressed as a R.S.D.% of a series of measurements. The experimental values obtained for the determination of LEF are presented in Table 2. The statistically evaluated results showed the R.S.D. values of 0.57–1.59% indicating good intra-day precision. Inter-day variability was calculated from assays on 3 days showed a R.S.D. of 1.41%. The R.S.D.% value is below 2% exhibiting the sufficient method precision and it is acceptable in analytical points' of view.

3.1.2. Linearity

Calibration curves for LEF were constructed by plotting concentration versus the ratio of peak area normalizations of LEF and IS and showed good linearity in the range of 2.7×10^{-6} to 5.5×10^{-5} M. Linear regression analysis of the curves was tabulated in Table 3. High correlation coefficients were obtained and the intercepts of the curves were not significantly different from zero.

Resolution was always good in the linearity range studied.

Certain analytical parameters such as limit of detection and limit of quantification values were calculated by computing the processed of integrated peak from HPLC chromatogram. LOD and LOQ values were estimated as [(standard deviation of repeatability)/(slope of regression equation)] by multiplying with 3.3 and 10, respectively. They were found to be 2.4×10^{-7} M for LOD and 7.2×10^{-7} M for LOQ.

3.1.3. Accuracy

Accuracy was tested as described in Section 2 and was evaluated as percentage error [(found concentration−spiked concentration)/spiked concentration] × 100%, and precision was evaluated by the coefficient of variation (C.V.%, R.S.D.%, [(S.D./mean) × 100]) with the confidence interval at the low, central and high concentration levels of linearity range. The acceptance criteria are not higher than 15% deviation from the nominal value for accuracy and not more than 15% C.V. for precision [16]. The percent recoveries were found almost 100% for drug substance and drug product and accuracy was much less than the acceptance criteria. The same concentration levels were used to evaluate precision as degree of repeatability. The values of R.S.D.% were also

Table 2
The results of the repeatability and the intermediate precision of LEF (5.4×10^{-6} M) calculated by the ratio of peak area normalization of LEF and IS

	Repeatability			Intermediate precision ($n = 6$, $k = 3$)
	First day ($n = 6$)	Second day ($n = 6$)	Third day ($n = 6$)	
Mean	0.3906	0.3976	0.3970	0.3950
S.D.	0.0046	0.0063	0.0022	0.0056
R.S.D.%	1.17	1.59	0.57	1.41
CL ($p = 0.05$)	0.3859–0.3954	0.3910–0.4042	0.3942–0.3999	0.3921–0.3979

Table 3
The calibration results of LEF determination related with statistical analysis at 0.5 ml min^{-1} flow rate and at 260 nm detection wavelength

	Intra-day			Inter-day
	Day 1 ($n = 6$)	Day 2 ($n = 6$)	Day 3 ($n = 6$)	Whole days ($n = 18$)
A (mean ± S.D.)	78590 ± 789	76830 ± 787	78530 ± 1008	77700 ± 503
B (mean ± S.D.)	0.003 ± 0.02	0.014 ± 0.02	−0.018 ± 0.02	0.007 ± 0.01
R	0.9998	0.9998	0.9998	0.9997
Sr	0.06	0.07	0.04	0.12
%R.S.D. of A	1.67	1.85	2.22	2.20
CL of A ($p < 0.05$)	±1251	±1353	±1663	±788

Abbreviations: A: slope; B: intercept; R: correlation coefficient; Sr: standard deviation of regression equation; R.S.D.: relative standard deviation; CL: confidence limits.

Table 4
The results of method accuracy of standard LEF and LEF spiked matrix

	Found LEF (M), (mean ± S.D., $n = 6$)	Recovery (%)	Accuracy (%)	R.S.D. (%)
Added LEF (M)				
5.5×10^{-6}	$5.62 \times 10^{-6} \pm 6.5 \times 10^{-8}$	100.4	2.37	1.16
2.7×10^{-5}	$2.81 \times 10^{-5} \pm 2.6 \times 10^{-7}$	101.2	2.21	0.93
5.5×10^{-5}	$5.69 \times 10^{-5} \pm 2.5 \times 10^{-7}$	103.7	3.69	0.44
LEF spiked matrix (M)				
5.4×10^{-6}	$5.30 \times 10^{-6} \pm 7.7 \times 10^{-8}$	97.96	2.18	0.91
2.7×10^{-5}	$2.69 \times 10^{-5} \pm 4.9 \times 10^{-7}$	98.73	1.53	1.09
4.2×10^{-5}	$4.29 \times 10^{-5} \pm 5.1 \times 10^{-7}$	102.40	2.61	1.46

much less than the acceptance criteria showing a good precision of the proposed method as seen in Table 4.

3.1.4. Specificity

In order to assure the specificity of the method on the C_{18} column three 2.0×10^{-4} M LEF standards were stressed with 0.1 N HCl (ambient temperature and 60 °C), 0.1 N NaOH (ambient temperature and 60 °C) and 3% (v/v) H_2O_2 (ambient temperature and 60 °C) for 15, 30, 45, 60, 90, and 120 min and injected into HPLC after 10-fold dilution with mobile phase. At both ambient and elevated temperatures LEF did not undergo degradation in acid solution or heating at 60 °C over 120 min. For the base stressed samples, degradation proceeds very rapidly at ambient temperature over 15 min. However, the H_2O_2 stressed sample at 60 °C showed approximately 20% degradation at 120 min, with degradation peak appearing at 2.04 min while the base stressed sample showed complete degradation at 15 min with one degradation peak appearing at 2.90 min as seen in Fig. 3a and b. None of these peaks seen in the H_2O_2 or base stressed samples showed any interference with LEF peak as confirmed by UV spectrum with a photodiode array detector.

Testing was also performed with the same column using tablet inactive ingredients to assure that these common tablet dosage form ingredients could be interfered with the peaks of interest. The data indicated that these ingredients did not

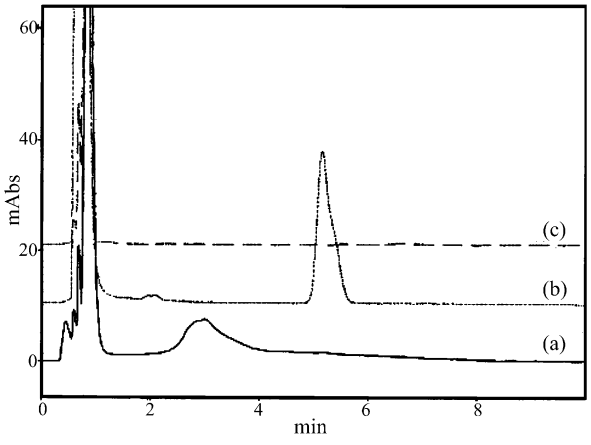

Fig. 3. Specificity of the method (a) LEF in 0.1 M NaOH; (b) LEF in 3% H_2O_2; (c) the matrix solution without LEF and IS.

interfere with LEF and IS peaks, so the specificity of this method was considered good as seen in Fig. 3c.

3.1.5. Application of the HPLC method to the LEF tablets

The application of the developed method for the determination of LEF was performed in tablets containing 20 mg active material as described in the experimental section. The peaks of tablet samples carried the characteristics of standard LEF and no interference originated from the matrix was observed. The content of a tablet was found to be 19.5 ± 0.2 (mean $\pm$ S.D., $n = 6$) and it is also in the limits of USP XXIV [17] suggestions.

The proposed method was compared to the UV spectrophotometry to verify the results obtained from HPLC. A calibration equation was obtained in the concentration range of 1.1×10^{-5}–3.3×10^{-5} M and at the wavelength of 260 nm by using 25% (v/v) ethanol as a blank. The relation between absorbance (A) and concentration of LEF (C) as molarity was [$A = 18544C\,(\mathrm{M}) - 0.0267$; $r = 0.9999$]. The tablet analysis results were found to be 19.4 ± 0.2 (mean $\pm$ S.D., $n = 6$) by UV spectrophotometry. High reproducibility and insignificant differences between the two methods were obtained at the 95% probability level for t- and F-test of significance of $1.75 < 2.57$ and $1.11 < 5.05$, respectively.

4. Conclusion

A simple and rapid HPLC method for the determination of LEF in tablets has been developed and validated. The retention time observed (5.2 min) allows us a rapid determination of the drug, which is important for routine analysis. The linearity range, limits of detection and quantification, precision, accuracy and specificity were processed to determine the suitability of the method and the confirmed results were obtained. HPLC has several superiorities compared with UV spectrophotometry such as smaller detection and quantification limits, small sample volumes and specificity. Specificity tests were successfully performed with HPLC and LEF could be well separated from the degradation products. Specificity of the method is also the superiority over FIA determination [12]. Thus, the developed HPLC method is rapid, specific, reliable and cost effective and can be proposed for routine analysis laboratories and quality control purposes.

Acknowledgement

The authors appreciate Research Council of Anadolu University for their support of our project.

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