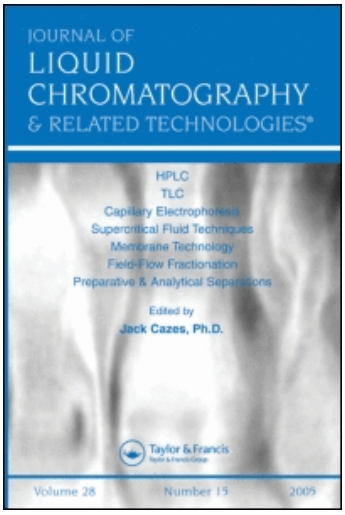

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Determination of Leflunomide in Pharmaceutical Tablets by Flow-Injection Analysis

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Abstract: A flow injection analysis (FIA) of leflunomide using UV-detection is described, in this study. The most suitable carrier solvent was found to be an aqueous solution of ethanol (25%, v/v). Leflunomide was determined at the optimum conditions, such as flow rate of $0.8\,mL \cdot min^{-1}$ and detection wavelength of 260 nm. The method has been validated and linearity was examined in the range of $2.75 \times 10^{-6} - 1.10 \times 10^{-4}$ M. The limit of detection (LOD) and quantitation (LOQ) were calculated to be 2.60×10^{-7} M (S/N = 3.3) and 7.87×10^{-7} M (S/N = 10), respectively. The application of the proposed method has been performed in pharmaceutical tablets of leflunomide and excellent results were obtained. The results were compared with those obtained from UV-spectrophotometry. Insignificant difference was found between the methods. As a result, the FIA method for the determination of leflunomide in pharmaceutical tablets can be proposed as a precise, accurate, sensitive, and cheap method for routine analysis laboratories.

Keywords: Leflunomide, Flow-injection analysis, Pharmaceutical analysis

INTRODUCTION

Leflunomide (LEF), [N-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide] is an novel isoxazol derivative with both anti-inflammatory and immunosuppressive properties. The chemical structure of LEF was

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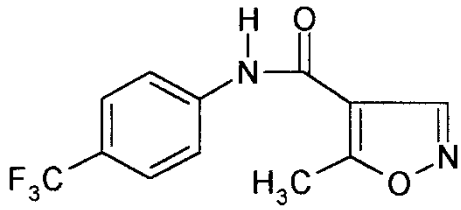

Figure 1. The chemical structure of LEF.

given in Figure 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 converted to its active metabolite, A77 1726. It is reported that A77 1726 possesses immunomodulator effects of the drug and is thought to inhibit cell proliferation of lymphocytes.[1]

Several high performance liquid chromatographic (HPLC) methods have been published for the kinetic monitoring and determination of the active metabolite of LEF, A77 1726, in human blood and plasma.[2–7] There are a few studies, including LC/MS/MS and affinity chromatography methods, to express the intercellular interactions of LEF.[8,9] According to the best of our knowledge, there is no study for the determination of LEF in pharmaceutical preparations and no reports concerning flow injection analysis (FIA) of LEF.

The aim of this study is to develop a simple, accurate, and precise FIA method for the determination of LEF in pharmaceutical tablets. After optimization of the experimental parameters, the method has been validated by investigating precision of peak response, linearity, accuracy, limit of detection, and limit of quantification. The proposed method has been applied to the analysis of pharmaceutical tablets of LEF, the results were compared with those obtained from UV-spectrophotometry and evaluated statistically.

EXPERIMENTAL

Chemicals

The standard LEF was obtained from Sigma (St.Louis, MO, USA). Its pharmaceutical tablet preparation of Arava®, a product from Aventis Pharma A.Ş. (Istanbul, TR) containing 20 mg active material, was purchased from a local drugstore. Other chemicals were of analytical grade and they were provided from Merck Co. (Darmstad, G). Double distilled water and ethanol used for the preparation of the solutions were produced in our laboratory using an all pyrex glass apparatus.

Apparatus

FIA was performed by a system consisting of models of LC 6A pump, SCL-6B auto injector, SPD-10A UV-visible variable wavelength detector, and CR-7A integrator all from Shimadzu (Kyoto, J). A model UV-2401 PC spectrophotometer from Shimadzu (Kyoto, J) for common spectrophotometric studies and a model of B-220 sonicator from Branson (California, USA) for sonication were also used.

Preparations of Solutions

The best carrier solvent used for FIA experiments was an aqueous solution of ethanol (25%, v/v). Stock solution of LEF was prepared at the concentration of 3.70×10^{-3} M in the carrier solvent mentioned above. The necessary dilutions were made from that stock solution for precision, linearity and accuracy studies.

Application of the Method to LEF Tablets

Ten Arava® tablets (each contained 20 mg LEF) were weighed, net weight of each tablet calculated, and finely powdered in a mortar. A sufficient amount of tablet powder equivalent to the average weight of the content of the tablet was accurately weighed and 10 mL ethanol was added to dissolve the active material. It was sonicated for 10 minutes and then the solution was centrifuged at 5000 rpm for 10 min. The supernatant was diluted as the standard solution to achieve the FIA determination.

RESULTS AND DISCUSSION

Optimization of the Method

Since the analytical measurements are realized in the form of solutions, the most convenient carrier solvent was investigated first. An aqueous carrier solvent system containing ethanol was preferred because of the solubility problem of LEF. There was not any precipitation in the aqueous solution of ethanol (25%, v/v). Therefore, it was accepted as a carrier solution in this study.

A 5.49×10^{-5} M LEF was prepared in the carrier solution and its UV absorbance spectrum was recorded in the range of 200–350 nm. It was observed that a maximum appeared at 260 nm. The detection of signals was performed at the mentioned wavelength.

The effect of flow-rate on the peak area response of LEF was investigated in the range of $0.1–3.0\,\text{mL}\cdot\text{min}^{-1}$. The peak morphologies of LEF signals

changed depending on the flow rate. Big and morphologically tailing peaks were observed at low flow rates, however, peak area at higher flow rates are gradually diminished. The plot of peak area of LEF versus flow rate exhibits a parabolic variation, which fits the equation of [(peak area)$^{-1} = 9.06 \times 10^{-6}$ (flow rate, mL $\cdot$ min^{-1}) -6.2×10^{-8}; r = 0.9998]. Optimum flow-rate exerting the peak symmetry and other quantitative evaluations were obtained at 0.8 mL $\cdot$ min^{-1}, and used in the rest of experiments.

The optimum FIA conditions were found to be a carrier solvent of an aqueous solution of ethanol (25%, v/v), flow rate of 0.8 mL $\cdot$ min^{-1}, and detection wavelength of 260 nm. The signals of LEF solutions recorded in the optimum conditions are shown in Figure 2.

Validation of the FIA Method

Peak Area Precision

The precision of the method was examined by injecting the LEF solutions which were prepared at three different concentrations (5.49×10^{-6} M,

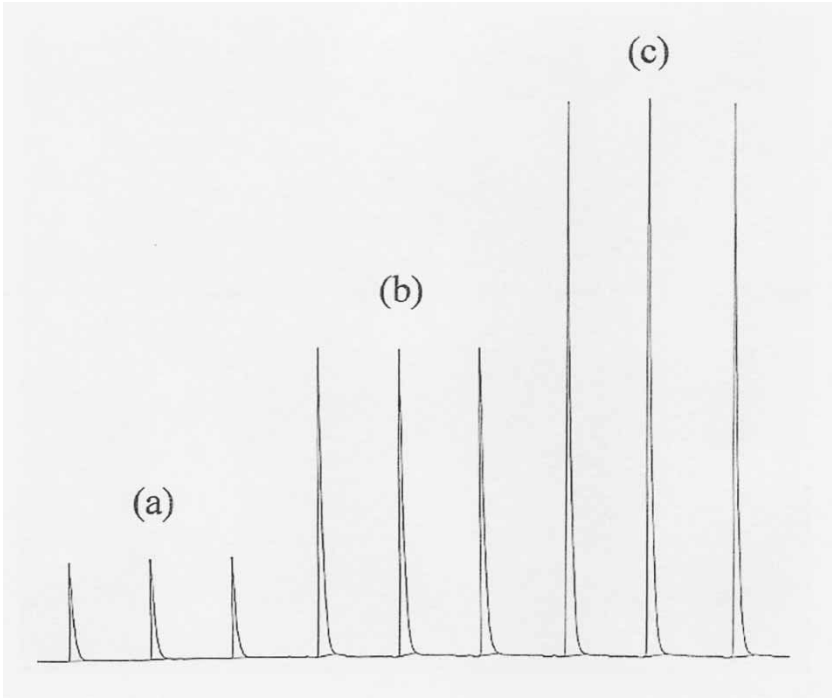

Figure 2. Triplicate signals of standard LEF solution recorded in the carrier solvent of aqueous solution of ethanol (25%, v/v) using 0.8 mL $\cdot$ min^{-1} flow rate and 260 nm UV-detection: (a) 2.75×10^{-6} M, (b) 5.49×10^{-6} M, and (c) 1.10×10^{-5} M.

1.10×10^{-5} M and 5.49×10^{-5} M). Each sets was injected eight times in a day consecutively and intra-day results were obtained. They were evaluated statistically relating to mean, standard deviation (SD), relative standard deviation (RSD %), and confidence limits (CL at $p < 0.05$) and illustrated in Table 1.

As seen from Table 1, the intra and inter-day precision decreases when the concentration increases. It can be attributed to the integration of the curves. At the level of 1.10×10^{-5} M or higher, the precision values are very low, even though inter-day precision values are lower than 2%, and it is accepted that they are under the analytical errors.[10,11,13]

Linearity

Linearity of the method in the concentration range of 2.75×10^{-6}–1.0×10^{-4} M was examined in the optimum conditions by injecting three sets, which were representing intra-days at five different concentrations. The results were shown in Table 2. Good linearity was obtained with high correlation coefficients and intercepts very close to the origin in the mentioned concentration range.

After getting the results of repeatability and linearity, it is possible to calculate the values of limit of detection (LOD) and limit of quantitation

Table 1. The intra-day and inter-day precision test (repeatability) of 5.49×10^{-6} M, 1.10×10^{-5} M and 5.49×10^{-5} M LEF as peak area in the carrier solvent of aqueous solution of ethanol (25%, v/v) using 0.8 mL · min^{-1} flow rate and 260 nm UV-detection

	Intra-day precision			Inter-day precision (n = 24)
	First day (n = 8)	Second day (n = 8)	Third day (n = 8)	
5.49×10^{-6} M LEF				
Mean (Area)	84,641	95,940	102,629	94,403.3
SD	1,560	3,353	4,909	3,548.5
RSD (%)	1.84	3.49	4.78	10.25
CL ($p < 0.05$)	±1,300.9	±2,796.1	±4,093.7	±2,959.2
1.10×10^{-5} M LEF				
Mean (Area)	152,251	155,705	153,712	153,889.3
SD	1,017	4,321	784.1	2,602.6
RSD (%)	0.67	2.77	0.51	1.94
CL ($p < 0.05$)	±848.1	±3,603.4	±653.9	±2,170.4
5.49×10^{-5} M LEF				
Mean (Area)	694,318	688,501	686,961	689,926.7
SD	7,887	5,450	4,879	6,210.5
RSD (%)	1.12	0.79	0.71	1.01
CL ($p < 0.05$)	±6,577.1	±4,544.9	±4,068.7	±5,179.1

Table 2. Calibration results of LEF (2.75×10^{-6}—1.0×10^{-4} M) in the optimum conditions

	Intra-day			Inter-day Whole days (n = 15)
	Day 1 (n = 5)	Day 2 (n = 5)	Day 3 (n = 5)	
A	1.28×10^{10}	1.27×10^{10}	1.25×10^{10}	1.27×10^{10}
B	1.12×10^{4}	1.62×10^{4}	2.09×10^{4}	1.61×10^{4}
R	0.9998	0.9998	0.9998	0.9998
Sr	2.44×10^{4}	3.22×10^{4}	2.71×10^{4}	5.25×10^{4}
RSD of Slope (%)	2.25	2.99	2.57	2.83
CL ($p < 0.05$)	$\pm 2.76 \times 10^{8}$	$\pm 3.63 \times 10^{8}$	$\pm 3.06 \times 10^{8}$	$\pm 1.65 \times 10^{8}$

Abbreviations: A: slope, B: intercept, R: correlation coefficient, Sr: standard deviation of regression equation, CL: confidence limits.

(LOQ). They can be achieved by multiplying [standard deviation of repeatability/slope of the calibration equation] with 3.3 and 10, respectively.[10,11,13] They were found to be 2.60×10^{-7} M for LOD and 7.87×10^{-7} M for LOQ.

Accuracy of the Method

The accuracy and precision, which corresponds to the effect of the inactive ingredients of the tablet formulation on the determination of LEF, was examined by preparing a synthetic excipient composition. It has been reported that an Arava® tablet contained colloidal silicon dioxide, crospovidone, hypromellose, lactose monohydrate, magnesium stearate, polyethylene glycol, povidone, starch, talc, titanium dioxide, and yellow ferric oxide as inactive ingredients;[12] so that the solution consisting of the inactive ingredients was prepared as the matrix solutions without LEF. Then, a certain amount of standard LEF solutions (1.46×10^{-5} M, 4.03×10^{-5} M, and 7.03×10^{-5} M) was spiked into each tube (n = 8) containing a synthetic mixture solution and they were shaken for a specific time. LEF solutions were then injected into the FIA system after centrifugation and the data was determined using a calibration equation. The accuracy was calculated as [(found concentration-spiked concentration)/spiked concentration] × 100%. The results are demonstrated in Table 3.

Table 3. The results of accuracy of LEF obtained by FIA (n = 8)

Added LEF (M)	Found LEF(M) (mean ± SD)	Recovery (%)	Accuracy (%)	RSD (%)
1.46×10^{-5}	$1.51 \times 10^{-5} \pm 2.75 \times 10^{-7}$	103.3	3.28	1.82
4.03×10^{-5}	$4.15 \times 10^{-5} \pm 6.79 \times 10^{-7}$	103.0	3.01	1.64
7.03×10^{-5}	$7.35 \times 10^{-5} \pm 6.24 \times 10^{-7}$	104.6	4.57	0.85

The acceptance criteria for the accuracy are not higher than 15% deviation from the nominal value and not more than 15% C.V. (coefficient of variation, RSD%) for precision.[13] As the accuracy and precision results obtained are in accordance with these criteria, it is concluded that the ingredients do not interfere with the proposed method.

Application of the FIA Method

The application of the developed method for the determination of LEF was performed in the pharmaceutical 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 19.3 ± 0.27 (mean $\pm$ SD, $n = 6$), and it is also in the limits of USP XXIV.[14]

The proposed method was compared to the UV-Spectrophotometry as a standard method.[15–21] First of all, a calibration equation was obtained in the concentration range of 1.10×10^{-5}–3.29×10^{-5} M and at the wavelength of 260 nm. The relation between absorbance (A) and concentration of LEF (C) as molarity was found to be [$A = 18544\ C\ (M) - 0.0267$; $r = 0.9999$].

The effect of the inactive ingredients given by the manufacturer of LEF tablets was also investigated by using common UV-spectrophotometry to verify the accuracy data of FIA method. In order to achieve this test, a matrix composition that is identical to those of LEF tablets was prepared and transferred to the tubes. A specific amount of standard LEF solution was added, left for a while on a stand, and shaken at times. Then, the procedure in the UV-spectrophotometry was followed. Determination of LEF in the synthetic mixture was realized and the results were demonstrated in Table 4.

The compared results for the tablet analysis of LEF obtained by FIA and UV-spectrophotometry are given in Table 5. High reproducibility and insignificant differences between FIA and UV-spectrophotometry were obtained at the 95% probability level.

Very few studies have been performed for the determination of LEF and chromatographic methods are not comparable to FIA because they are not included in the pharmaceutical tablet analysis. It is known that FIA is a very convenient method for the active drug content in the pharmaceutical analysis. It has superiorities regarding simplicity, versatility, high sampling

Table 4. The results of accuracy of LEF realized by UV-spectrofotometry ($n = 8$)

Added LEF (M)	Found LEF(M) (mean $\pm$ SD)	Recovery (%)	Accuracy (%)	RSD (%)
1.46×10^{-5}	$1.56 \times 10^{-5} \pm 9.0 \times 10^{-8}$	106.70	6.70	0.58
4.03×10^{-5}	$4.13 \times 10^{-5} \pm 9.7 \times 10^{-8}$	102.51	2.51	0.24
7.03×10^{-5}	$7.20 \times 10^{-5} \pm 4.0 \times 10^{-7}$	102.50	2.50	0.56

Table 5. The results of tablet analysis of LEF performed by FIA and UV-spectrophotometry.

	FIA	UV-spectrophotometry
Mean (n = 6)	19.30	19.40
SD	0.27	0.19
RSD (%)	1.41	0.99
t-test ($p < 0.05$)	0.15	Table $t_{0.05} = 2.57$
F-test ($p < 0.05$)	0.49	Table $F_{0.05} = 5.05$

frequency, degree of automation, and low expense of reagents and samples providing the technique suitable for satisfying the increasing demand for control and routine analysis in many fields of analytical chemistry.[22] Our validated FIA method confirmed the mentioned properties for the determination of LEF in pharmaceutical tablets without any ingredient effect on active material.

In conclusion, the FIA method reported in this study can be practically used for the direct determination of LEF in laboratories as a rapid, accurate, reliable, and sensitive method.

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