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Determination of zafirlukast, a selective leukotriene antagonist, human plasma by normal-phase high-performance liquid chromatography with fluorescence detection

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Abstract

A high-performance liquid chromatographic (HPLC) method was developed for the determination of zafirlukast, a selective peptide leukotriene receptor antagonist, in human plasma. Zafirlukast and the internal standard, ICI 198 707, were extracted from deproteinated plasma samples using large reservoir C_{18} solid-phase extraction columns and analyzed by normal-phase liquid chromatography with fluorescence detection. The method had a lower limit of quantitation of 0.75 ng/ml and a linear calibration curve in the range of 0.75 to 200 ng/ml. The absolute recovery of zafirlukast was >90%, and the within-day and between-day relative standard deviations were <9%. The utility of the method in the characterization of the plasma concentration–time profiles of zafirlukast in clinical studies was demonstrated. © 1997 Elsevier Science B.V.

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1. Introduction

Zafirlukast [4-5(-cyclopentyloxycarbonylamino-1-methylindol-3-ylmethyl)-3-methoxy-*n*-*o*-tolylsulfonylbenzamide], a selective peptide leukotriene receptor antagonist, is being developed by ZENECA Pharmaceuticals for use in the treatment of asthma. In the United States, zafirlukast has been approved by the FDA for use in the prophylaxis and chronic treatment of asthma in adults and children 12 years and older. To support the evaluation of the pharmacokinetics of zafirlukast in man, a sensitive, accurate and precise HPLC method for the determination of zafirlukast in human plasma was developed. Validation data and salient development features of the method are summarized in this paper. The method was successfully used to monitor zafirlukast plasma concentrations in more than fifty clinical trials.

2. Experimental

2.1. Reagents and standards

Zafirlukast and the internal standard, ICI 198 707 [4-5(-cyclopentyloxycarbonylamino-1-methylindol-3-ylmethyl)-3-methoxy-*n*-benzylsulfonylbenzamide] were supplied by the Analytical Development and

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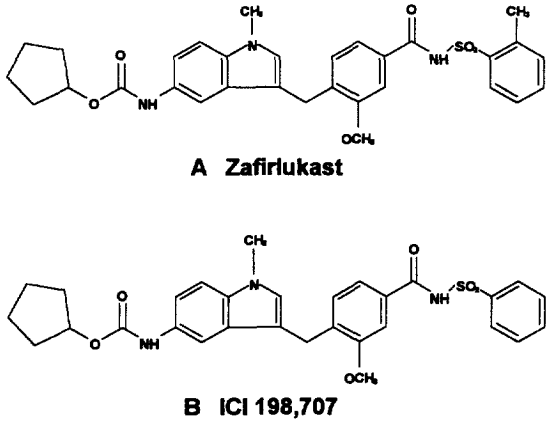

Fig. 1. Chemical structure of (A) zafirlukast and (B) ICI 198 707.

Automation Department of ZENECA Pharmaceuticals. The chemical structure (see Fig. 1) and purity of the two standards were certified by the department using high-performance thin layer chromatography (HPTLC), HPLC, infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (NMR) and mass spectroscopy (MS). All other reagents and solvents used were either analytical- or HPLC-grade.

2.2. Instrumentation

The HPLC system consisted of a Hewlett-Packard HP-1090 liquid chromatograph (Hewlett-Packard, Wilmington, DE, USA) equipped with an integrated automatic sampler and a Jasco 821 FPS fluorescence detector (Jasco, Easton, MD, USA). Chromatographic data handling was performed with a VG Multichrom Data Acquisition System (VG Laboratory Systems, Altrincham, UK).

2.3. Chromatographic conditions

Chromatographic analysis was performed on a 5 μm 150×4.6 mm I.D. Zorbax CN analytical column (Mac-Mod Analytical, Chadds Ford, PA, USA) protected with a 5 μm 12.5×4.0 mm Zorbax CN guard column (Mac-Mod Analytical) at room temperature. The mobile phase was tetrahydrofuran (THF)–hexane–90% glacial acetic acid in water (300:700:1, v/v). The flow-rate was 0.9 ml/min and the injection volume was 150 μl. Fluorescence detection was performed with an excitation wavelength of 250 nm and an emission wavelength of 452 nm.

2.4. Preparation of standards

Stock spiking solutions of zafirlukast were prepared in acetonitrile at concentrations of 100 and 200 μg/ml. Appropriate dilutions of the spiking solutions were made in acetonitrile to prepare analytical standard solutions. Matrix calibration standards (0.75, 2.50, 10.0, 20.0, 42.0, 80.0 and 200 ng/ml) were prepared daily by spiking control plasma with zafirlukast. The quality control (QC) samples (2.00, 40.0 and 100 ng/ml) were prepared in a batch and aliquots were stored at −20°C until analyzed. A stock spiking solution of internal standard (ICI 198 707) was prepared in THF at a concentration of 36 μg/ml. All samples, except blank plasma samples, were spiked with 25 μl of internal standard spiking solution prior to sample preparation and analysis.

2.5. Sample preparation

After spiking with the internal standard solution, 1 ml of plasma sample was mixed with 1 ml of acetonitrile and centrifuged at 1000 *g* for approximately 5 min. The supernatant was then decanted and 5 ml of 0.01 *M* triethyl amonium phosphate buffer (pH 7.0) added. A large reservoir Bond Elute (LRBE) C_{18} column (Varian, Harbor City, CA, USA) was conditioned by sequentially drawing methanol (2×1 ml) and water through the column (2×1 ml). The diluted supernatant was passed through the extraction column and the eluate discarded. Zafirlukast was eluted into a 6 ml polypropylene tube with three separate aliquots of a 0.5 ml solution of tetrahydrofuran–acetonitrile–triethylamine (10:90:0.2, v/v). The solvent was evaporated under nitrogen at 40°C. The sample was reconsti-

tuted in 250 μl of the mobile phase, filtered with a 0.45 μm syringe filter, and transferred to an HPLC minivial.

3. Results and discussion

3.1. Method development

A reversed-phase HPLC method with ultraviolet (UV) detection has been developed for the determination of zafirlukast in plasma samples of animals [1]. This method, although more than suitable for use in support of pharmacokinetic and safety evaluation studies in animals, lacks adequate selectivity and sensitivity to support the characterization of zafirlukast pharmacokinetics in man. Hence, alternative means of detection were explored to address the need for additional selectivity and sensitivity.

Zafirlukast contains both a sulfonamide group and an indole group (see Fig. 1A) which could be capitalized on for detection. Photoconductivity detection, although reported to give sensitive responses to sulfonamides in serum and urine samples [2,3], did not provide adequate sensitivity to zafirlukast. Fluorescence from compounds containing the indole functional group has been observed [4] although the fluorescence intensities are weak when solvents that could hydrogen bond with the analyte are used [5]. Indeed, relative to the fluorescent signal intensity of zafirlukast in acetonitrile, the incorporation of protic solvent/acetonitrile solvent pairs resulted in a significant drop in both sensitivity (signal-to-noise ratio) and selectivity (as measured by the Stoke's shift). This implied that aqueous mobile phases should be avoided and an increase in both selectivity and sensitivity could be gained using normal-phase chromatography. In fact, when minimizing or avoiding both protic solvents and chlorinated solvents (which probably quench the fluorescence by increasing the probability for intersystem crossing to the triplet state [6]), the sensitivity obtained for zafirlukast via fluorescence detection surpassed that of UV detection. Chromatographic and fluorescence signal optimization resulted in the chromatographic system listed in Section 2.3.

3.2. Chromatography

Examples of chromatograms obtained from control plasma, spiked plasma and dosed plasma extracts are shown in Fig. 2. Zafirlukast eluted at approximately 9 to 10 min and the internal standard, ICI 198 707 (see Fig. 1B), eluted at approximately 10 to 11 min. Since the method was first used (1988), the retention times of the analyte and the internal standard have been consistent within- and between-runs and between-columns.

3.3. Calibration curve and linearity

The daily calibration curves were performed by spiking blank plasma samples with zafirlukast at 0.75 to 200 ng/ml and are best described by the following power fit equation $y=mx^b$, where y=spiked concentration, x=peak area ratio of zafirlukast to internal standard, m=factor and b=exponent.

To fit the above equation, an indirect fitting algorithm was employed in which the above equation was linearized via log transformation and analyzed by linear regression. Besides being simple to implement, this algorithm helped stabilize variance by transforming errors to a more homogeneous scale, thus eliminating the need for a weighted fit.

The factor, exponent and correlation coefficient for eight consecutive analytical runs are summarized in Table 1. The regression parameters characterizing the calibration curves were very reproducible between runs. In addition, the exponent values of the calibration curves from all the runs were not significantly different from 1 ($p>0.05$), indicating linearity within the calibrated range. Excellent correlation between spiked concentration and response ($r^2 \geq 0.999$) was observed in all calibration curves.

3.4. Precision and accuracy

The within-day and between-day precision and accuracy of the assay were evaluated by assaying duplicate quality control samples spiked at three different concentrations (2.00, 40.0 and 100 ng/ml) on eight consecutive days. The within-day and between-day variations, as determined by analysis of variance (ANOVA), were all <9% R.S.D. at all three

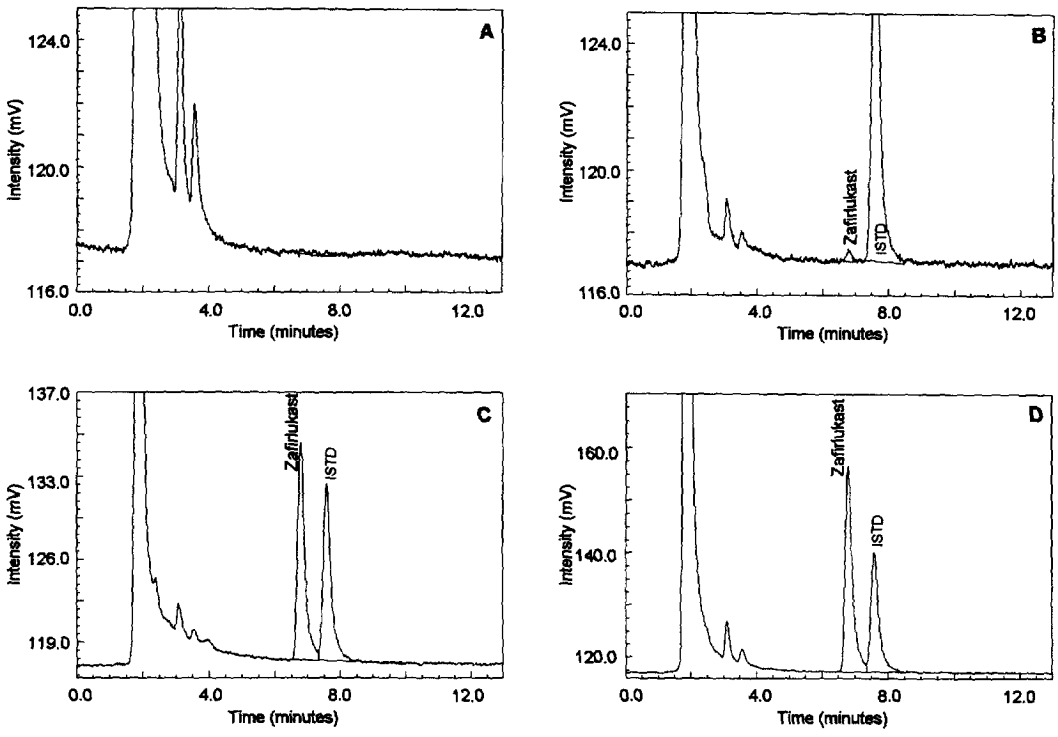

Fig. 2. Chromatograms from plasma extracts: (A) control plasma sample; (B) plasma sample spiked with 0.75 ng/ml of zafirlukast (lower limit of quantitation); (C) plasma sample spiked with 40 ng/ml of zafirlukast; (D) human plasma sample, 30 min after 20 mg oral dose of zafirlukast. ISTD=internal standard.

Table 1
Regression parameters and statistics of the calibration curves

Run	Factor (m)	Exponent (b)	Correlation coefficient (r^2)
1	36.9	1.000	0.999
2	37.5	0.992	1.000
3	40.3	0.963	0.999
4	43.3	1.050	0.999
5	43.9	0.996	1.000
6	38.6	0.995	0.999
7	41.4	1.020	1.000
8	43.4	0.975	0.999
Mean	40.7	0.999	1.000
S.D.	2.78	0.270	<0.0001
%R.S.D.	6.89	2.79	0.007

concentrations (see Table 2). The low, mid and high quality controls samples averaged 95.0, 100.0 and 95.5% of their theoretical values, respectively.

3.5. Sensitivity

The lower limit of quantitation was 0.75 ng/ml (Fig. 2B) and was defined as the smallest concentration included in the standard curve [7]. Samples spiked at the lower limit of quantitation produced a peak height response with signal-to-noise ratio of at least 8:1 and, when quantified against the calibration curves, consistently had concentrations within 20% of theory.

3.6. Recovery

The absolute recovery of zafirlukast was evaluated by comparing the peak areas of the spiked calibration

Table 2
Precision and accuracy of the method

Concentration (ng/ml)	n	Mean	Within-day variation (%R.S.D.)	Between-day variation (%R.S.D.)	Accuracy (%)
2.00	13	1.9	6.8	5.9	95.0
40.0	13	40.1	8.7	3.9	100.0
100	13	95.5	8.1	3.9	95.5

standards against those of the analytical standards on three validation days. The recovery of plasma calibration standards was constant over the calibration range of 0.75 to 200 ng/ml (see Table 3) and averaged 92% (10% R.S.D.).

3.7. Specificity

The method was shown to be specific against acetaminophen, acetylsalicylic acid, caffeine, ibuprofen and metabolites formed within 1 h of taking the aforementioned drugs. In addition no interferences were noted in samples from cigarette smokers, samples containing benzyl alcohol and albuterol, or in hemolyzed samples. All identified circulating metabolites of zafirlukast in man did not interfere with the quantitation of the parent drug in this method.

3.8. Stability

The stability of zafirlukast when frozen in plasma at −70°C and −20°C, respectively was established for at least one year and three months. In addition, zafirlukast remained stable in plasma after three freeze–thaw cycles. Plasma samples spiked with zafirlukast and ICI 198 707 remained stable when stored for 1.5 h in the extraction solvent prior to completion of sample processing. Zafirlukast analytical standard solutions were stable for at least two months when stored at 4°C in tightly capped polypropylene tubes.

3.9. Application of method

Fig. 3 shows the typical profile of zafirlukast concentrations in plasma of a healthy volunteer after oral administration of a single dose of 20 mg of

Table 3
Absolute recovery of zafirlukast from plasma

Concentration (ng/ml)	Recovery (%)						
	Day 1		Day 2		Day 3		
	Assay 1	Assay 2	Assay 1	Assay 2	Assay 1	Assay 2	Pooled
0.75	70	109	79	85	96	85	
2.50	105	95	101	87	109	87	
10.0	99	104	83	94	82	94	
20.0	105	99	85	81	101	91	
42.0	87	90	91	82	92	97	
80.0	82	83	86	92	107	102	
200	83	87	87	93	94	–	
Mean	93		88		91		92
%R.S.D.	12		6.8		8.5		10
S.E.M.	3.0		1.6		2.2		1.4

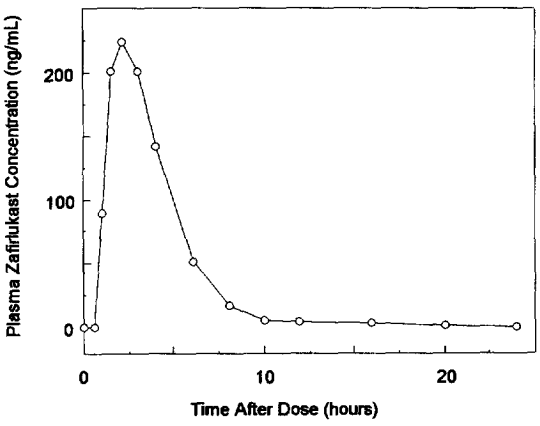

Fig. 3. Plasma concentration–time profile following oral administration of a single 20 mg dose of zafirlukast to a healthy volunteer.

zafirlukast. At this dose level, this method was capable of quantifying zafirlukast plasma concentrations which were less than 0.5% of peak levels and monitoring zafirlukast plasma concentrations for at least 24 h after dosing.

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