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Rapid determination of the active leflunomide metabolite A77 1726 in human plasma by high-performance liquid chromatography

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Abstract

A simple method for the measurement of the active leflunomide metabolite A77 1726 in human plasma by HPLC is presented. The sample workup was simple, using acetonitrile for protein precipitation. Chromatographic separation of A77 1726 and the internal standard, α-phenylcinnamic acid, was achieved using a C_{18} column with UV detection at 305 nm. The assay displayed reproducible linearity for A77 1726 with determination coefficients (r^2) > 0.997 over the concentration range 0.5–60.0 μg/ml. The reproducibility (%CV) for intra- and inter-day assays of spiked controls was <5%. The limit of quantification was 0.8 μg/ml. The average absolute recovery was approximately 100%. This assay is suitable for the determination of A77 1726 in plasma of patients taking leflunomide, and is simpler to use than other HPLC methods reported previously.

Keywords: Leflunomide; A77 1726

1. Introduction

Leflunomide [*N*-(4-trifluoromethylphenyl)-5-methylisoxazole-4-carboxamide] is a disease modifying antirheumatic drug (DMARD) with immunomodulatory activity. It is indicated for the treatment of active rheumatoid arthritis to reduce signs and symptoms, and to retard structural damage as measured by radiographic erosions and joint-space narrowing [1,2]. The immunomodulating effect of leflunomide is expressed after rapid conversion to its pharmacologically active metabolite, a malononitrilamide A77 1726 [2-cyano-3-hydroxy-*N*-(4-trifluoromethylphenyl)-2-butenamide] (Fig. 1), upon absorption [1,3]. A77 1726 affects de novo pyrimidine synthesis by inhibition of the enzyme dihydroorotate dehydrogenase (DHODH), thereby preferentially causing cell cycle arrest of autoimmune lymphocytes [4]. Since the conversion of leflunomide to A77 1726 in vivo is essentially complete [1], most pharmacokinetic studies have appropriately been conducted measuring A77 1726 and not leflunomide.

Previously published studies describing the measurement of A77 1726 by high-performance liquid chromatography (HPLC) have utilised a gradient system on 25–45% acetonitrile to achieve separation of A77 1726 from leflunomide [5]. Besides the instrumentation needed for gradient operation, this can be time consuming as a washout period is required between runs to re-equilibrate the column and return to a stable baseline. Various extraction techniques for sample clean up have been tried, including extraction with ethyl acetate in two separate steps prior to reconstitution [6], and vortex-mixing the treated sample for 1 h following addition of the extraction solvent [7]. Methods involving tandem C_8 columns maintained at 70 °C have also been used for the chromatographic analysis of A77 1726 [8].

A more recent paper describing the HPLC analysis of both A77 1726 and leflunomide also utilises temperature control on column [9]. However, this requires more complex hardware, a procedure not easily replicated in some laboratory settings. Additionally, the investigators selected warfarin as the internal standard [9]. However, there is a potential for peak interferences as warfarin is commonly prescribed as an oral anticoagulant.

The present study describes a rapid, sensitive and selective method for the determination of A77 1726 in human plasma using conventional HPLC instrumentation. It comprises of a

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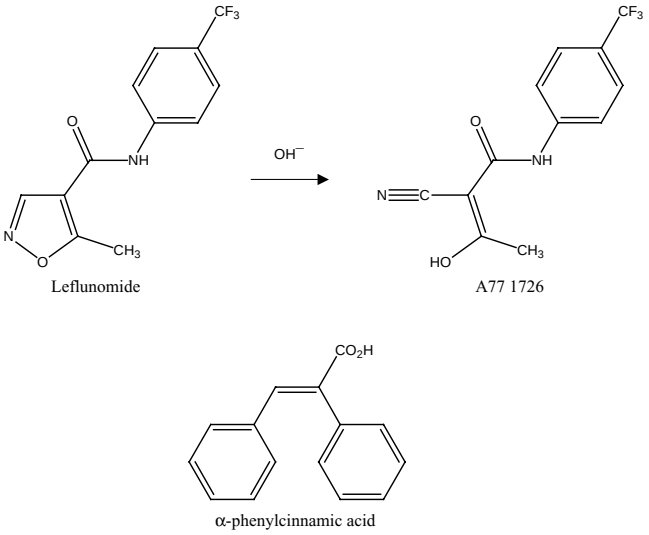

Fig. 1. Chemical structures of leflunomide, A77 1726 and α-phenylcinnamic acid (internal standard).

simple protein precipitation step, followed by HPLC analysis with ultraviolet (UV) detection.

2. Experimental

2.1. Reagents and chemicals

Research grade A77 1726 (99.9% purity), the active metabolite of leflunomide, was obtained as a gift (Aventis Pharma Global Pharmaceutical Development, Germany). The internal standard, α-phenylcinnamic acid, was purchased from Sigma (Sydney, NSW, Australia). Reagent grade anhydrous sodium acetate was obtained from Scharlau (Barcelona, Spain). LAB-SCAN acetonitrile (Bangkok, Thailand) and Mallinckrodt ChromAR® methanol (Kentucky, USA) were all HPLC grade. Glacial acetic acid (BDH AnalaR®, Kilsyth, VIC, Australia), and di-sodium hydrogen orthophosphate (Fronine Pty Ltd., Riverstone, NSW, Australia) were all analytical grade chemicals. Water used for all experiments was deionised and was degassed and passed through a microbiological filter membrane (0.45 μm pores) before use. Drug-free human plasma (past the "use-by" date) was obtained from the Red Cross Blood Bank (Brisbane, Qld, Australia).

Di-sodium hydrogen orthophosphate (0.05 M) was adjusted to pH 8.00 ± 0.02. This buffer was used to prepare stock solutions of A77 1726 in 10% (v/v) methanol.

2.2. Standard preparation

A stock solution of A77 1726 was prepared at a concentration of 500.0 μg/ml, and then serially diluted with phosphate buffer (pH 8, 0.05 M) to give working standard solutions of 5.0, 10.0, 25.0, 50.0, 100.0, and 250.0 μg/ml. A working internal standard solution of α-phenylcinnamic acid (5 mg/ml) was prepared in methanol. Stock solutions and standards were all stored in glass amber vials at approximately 5 °C.

2.3. Instrumentation

The HPLC system comprised of a Shimadzu LC-10AD pump (Shimadzu, Kyoto, Japan) equipped with a Shimadzu SIL-10AXL autoinjector (Shimadzu, Kyoto, Japan), and a Shimadzu SPD-10A UV-Vis detector (Shimadzu, Kyoto, Japan). The system was controlled with Class-LC10 software through a Shimadzu CBM-10A controller (Shimadzu, Kyoto, Japan). Chromatographic separation was performed on a Waters (Waters, Milford, MA, USA) Nova-Pak C_{18} column (4 μm spherical particles, pore size 60 Å, 3.9 mm i.d. × 150 mm), protected by a Waters Nova-Pak C_{18} Sentry guard column (4 μm, 3.9 mm i.d. × 20 mm) and an in-line high-pressure column prefilter SSI (2 μm, 1.5 mm) (Alltech, Deerfield, IL, USA) in front of the guard column.

2.4. HPLC

The isocratic mobile phase (35% acetonitrile–acetate buffer) was prepared by adding 650 ml of 0.05 M sodium acetate buffer (adjusted to pH 2.50 ± 0.02 with glacial acetic acid) to 350 ml acetonitrile, followed by degassing and filtration (0.45 μm pores) under negative pressure. The column flow rate was 1.5 ml/min and the detector set to 305 nm.

2.5. Sample preparation

Calibration standards and quality controls were prepared over the A77 1726 concentration range of 0.5–60.0 μg/ml by adding the appropriate volume of working standard solutions to a clean 1.5 ml plastic micro tube and making up to 500 μl with blank plasma, followed by the addition of 10 μl of working internal standard solution, and the mixture vortexed for 5 s. Calibrators, controls or unknowns (100 μl) were transferred to 1.5 ml plastic tubes, and acetonitrile (200 μl) added to each. Following vortex-agitation (30 s), the tubes were left to stand at ambient temperature for 15 min, and then centrifuged (approximately 5000 × *g*, 20 min). A 50 μl sample of the clear supernatant fluid was injected on to the column.

2.6. Assay validation

A seven point calibration standard curve in plasma was generated on five separate assay occasions. Five replicates of spiked controls, at three concentrations, were included with each validation day of analysis to assess assay variability.

The absolute recovery of the method was determined by comparing the peak areas of spiked quality control samples with samples of the same target concentrations made up in mobile phase. Stability was investigated on spiked control

samples subjected to two and three freeze-thaw cycles and on samples stored at ambient temperature (approximately 22 °C) for 24 h.

The selectivity of the method for endogenous peaks was established by analysing drug-free plasma samples obtained from six people (Red Cross Blood Bank). A comparison of these chromatograms with those obtained after spiking plasma blank with A77 1726 and internal standard ascertained that endogenous substances do not interfere with the assay. The potential for interference from other medication was assessed by inspecting the chromatograms of a number of drugs which are often prescribed for patients taking leflunomide.

2.7. Quantitation

Peak area ratios of A77 1726 to internal standard generated from a seven point standard curve were determined on five days. Linearity of the curve over the concentration range 0.5–60.0 μg/ml A77 1726 was determined using regression analysis.

Intra-day, inter-day and total precision of the assay was calculated as the coefficients of variation (%CV) using a program written by one of us (BC), based on theory described previously [10]. The intra-day precision was determined from plasma standards in five replicates at three different levels (0.8, 3.0, 40.0 μg/ml). The inter-day precision was calculated for each of the three concentration levels (0.8, 3.0, 40.0 μg/ml) generated over the five validation days. Accuracy of the assay was determined by comparing the back-calculations of the calibration standards and quality controls to their known values.

Criteria for acceptance of an analytical run [11] were that the determination coefficient (r^2) must be ≥0.995; back-calculations for the calibrators must be ±10% of the respective target concentrations, and three of the five quality controls must be within 15% of the theoretical concentration (where each concentration must be represented).

3. Results and discussion

Both A77 1726 and α-phenylcinnamic acid were well resolved from each other and from other peaks in the matrix. There were no endogenous substances in the plasma that interfered with the analytical runs, as shown in Fig. 2a and b. The retention times for A77 1726 and the internal standard averaged 2.2 min and 5.7 min respectively. Linearity of the A77 1726 standard curve at the concentrations of 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, and 60.0 μg/ml was determined by using peak area ratios of A77 1726 to α-phenylcinnamic acid, versus A77 1726 concentrations. Linear regression consistently produced r^2 values greater than 0.997. Slope and intercept were 0.015 ± 0.001 and 0.002 ± 0.004, respectively. Back-calculated concentrations from peak area ratios were within 10% of the theoretical target concentrations. Table 1 shows the inter-day accuracy and precision of the calibration standards over five validation occasions.

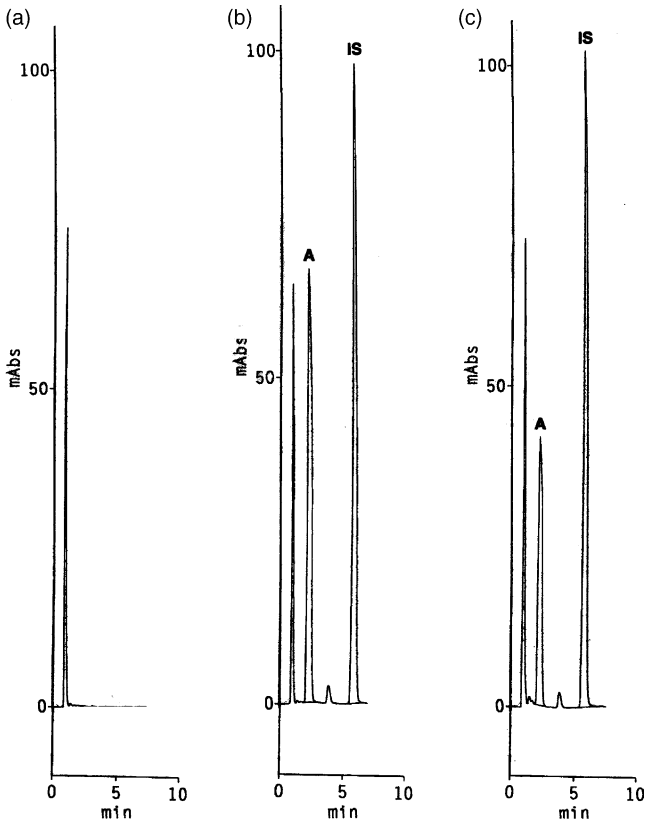

Fig. 2. (a) Chromatogram of blank plasma, (b) chromatogram of A77 1726 quality control sample (40.0 μg/ml), and the internal standard α-phenylcinnamic acid (100.0 μg/ml), (c) chromatogram of a patient sample, containing A77 1726 (21.4 μg/ml) and the internal standard (100.0 μg/ml). The patient was on 20 mg leflunomide daily, with the sample taken 24 h following the dose. Peaks labelled A and IS correspond to A77 1726 and the internal standard, respectively. The small peak observable in (b) and (c) is a minor impurity in the internal standard.

Quality control plasma samples spiked with 0.8, 3.0 and 40.0 μg/ml of A77 1726 were evaluated (Table 2). The accuracy was 99.4, 100.1, and 100.4%, respectively. The coefficient of variation for intra- and inter-day precision of

Table 1
Inter-day accuracy and precision of A77 1726 calibration standards in human plasma

Target (spiked) concentration (μg/ml)	Mean ± S.D.[a] ($n = 5$) (μg/ml)	Accuracy (%)[b]	Imprecision (%CV)
0.5	0.507 ± 0.035	101.4	6.9
1.0	1.01 ± 0.04	100.8	3.5
2.5	2.47 ± 0.07	98.7	3.0
5.0	5.01 ± 0.03	100.2	0.61
10.0	10.1 ± 0.1	101.0	0.85
25.0	25.3 ± 0.9	101.2	3.6
60.0	59.9 ± 0.3	99.8	0.55

%CV, coefficients of variation.
[a] Back-calculated plasma concentrations.
[b] [Mean assayed concentration − nominal concentration]/[nominal concentration] × 100.

Table 2
Precision data of A77 1726 quality controls in human plasma

QC (spiked) concentration (μg/ml)	Mean ± S.D.[a] (μg/ml)	Imprecision (%CV)		
		Intra-day	Inter-day[b]	Total
0.8	0.795 ± 0.019	2.4	0.54	2.5
3.0	3.00 ± 0.103	3.5	1.0	3.7
40.0	040.2 ± 1.56	3.1	2.6	4.0

QC, quality control; %CV, coefficients of variation.
[a] Back-calculated plasma concentrations.
[b] Five replicates over five validation occasions.

the quality controls was <5% for all three concentrations (Table 2). The data suggest that the method was both accurate and precise for analytical purposes. The limit of quantitation was nominally set to 0.8 μg/ml, although there was scope for this to be lower if needed in view of the results obtained for accuracy and precision at the lowest spiked control.

The mean absolute recovery at 0.8, 3.0, 40.0 μg/ml (n = 5 at each concentration) was 101.3% for A77 1726 and 100.0% for α-phenylcinnamic acid, indicating an excellent quantitative recovery of analyte and the internal standard.

A77 1726 was verified to be stable in plasma following a comparison of the results of analysing samples containing 0.8, 3.0, and 40.0 μg/ml (n = 3 at each concentration), which were subjected to two and three cycles of freezing (−20 °C) and subsequent thawing, with the third set being stored at ambient temperature for 24 h prior to analysis. There was excellent agreement among the results for the three treatments as seen in Table 3. However, it was noted that plasma A77 1726 stored at ambient temperatures, as opposed to immediate storage in freezer, exhibits weaker stability.

A number of non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroids may be administered concomitantly with leflunomide in the treatment of rheumatoid arthritis. Prednisone (1.7 min), prednisolone (1.8 min), indomethacin (10.1 min), naproxen (3.8 min), celecoxib (did not elute), and ibuprofen (did not elute) were found not to interfere with the assay. In addition, under the validated conditions, leflunomide was also elucidated (4.5 min) and did not interfere with the assay. It was not incorporated into the method due to its absence in plasma samples of patients.

The assay sample cleanup step was initially investigated using 10% (w/v) trichloroacetic acid as the protein precipitant instead of acetonitrile. While this was satisfactory in terms of selectivity, accuracy and imprecision, the recovery was very poor at 6.6 and 9.9% for A77 1726 and the internal standard, respectively. We are unsure of the reason for this except to speculate that there may be non-specific occlusion of the two compounds in the precipitated proteins.

Table 3
Stability data of A77 1726 quality controls in human plasma

QC (spiked) concentration (μg/ml)	Stability	Mean ± S.D.[a] (n = 3) (μg/ml)	Accuracy (%)[b]	Imprecision (%CV)
0.8	2 F/T	0.778 ± 0.018	97.3	2.3
	3 F/T	0.814 ± 0.015	101.8	1.9
	24 h	0.761 ± 0.004	95.1	0.48
3.0	2 F/T	3.03 ± 0.30	100.8	10
	3 F/T	3.09 ± 0.03	103.1	0.91
	24 h	2.84 ± 0.01	94.8	0.23
40.0	2 F/T	43.4 ± 4.01	108.5	9.2
	3 F/T	40.3 ± 1.72	100.7	4.3
	24 h	37.5 ± 0.04	93.8	0.11

QC, quality control; %CV, coefficients of variation; F/T, freeze/thaw cycle.
[a] Back-calculated plasma concentrations.
[b] [Mean assayed concentration − nominal concentration]/[nominal concentration] × 100.

4. Conclusion

A rapid, sensitive, selective, accurate and precise HPLC method has been developed, which is suitable for the determination of A77 1726 in the plasma of patients taking leflunomide, and which is simpler to use than other HPLC methods reported previously. The simplicity of the protein precipitation pre-treatment method resulted in reduced assay time without compromising assay performance. The method is accurate over the range 0.8–60.0 μg/ml for A77 1726 in plasma, which encompasses the concentrations achieved in plasma following clinical doses of either 10 mg or 20 mg of leflunomide [12,13]. The assay is currently being used in a study of the population pharmacokinetics of leflunomide in patients with rheumatoid arthritis.

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