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A rapid and simple determination of A77 1726 in human serum by high-performance liquid chromatography and its application for optimization of leflunomide therapy

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Abstract

Leflunomide is a disease-modifying antirheumatic drug, which is bioactivated by formation of A77 1726. In this study a rapid and simple quantitative assay using a reversed phase HPLC-UV method is validated for detection of A77 1726 in human serum. The HPLC-UV method uses a mobile phase consisting of methanol and a KH_2PO_4-buffer (45 mM, pH = 3) (50:50,v/v), at a flow rate of 1 mL/min. A77 1726 is detected by UV-absorption at 295 nm with a retention time of 8.9 min. Demoxepam is used as internal standard. Validation showed lower and upper limits of quantitation of 0.5 and 100 mg/L, respectively. The assay was linear over the concentration range of 0.5–100 mg/L (r^2 > 0.999). Intra- and inter-day precision showed coefficients of variation within 15% over the complete concentration range; accuracy was within 8%. Commonly prescribed drugs to treat rheumatoid arthritis like disease-modifying antirheumatic drugs, analgesics and corticosteroids, and their main metabolites, are separated from A77 1726 with a resolution >2. Serum levels of A77 1726 in 37 patients on leflunomide therapy were determined using this HPLC-UV method. Measured serum A77 1726 serum concentrations in patient samples showed large variability with a range of 3–176 mg/L.

Keywords: Leflunomide; A77 1726; High-performance liquid chromatography; Therapeutic drug monitoring

1. Introduction

Leflunomide is a disease modifying antirheumatic drug of the isoxazole class. After oral administration leflunomide is rapidly, non-enzymatically and completely converted into its long-acting, active metabolite A77 1726 (2-cyano-3-hydroxy-N-(4-trifluoromethylphenyl)-crotonamide; Fig. 1) [1]. A77 1726 has antirheumatic activity through inhibition of the enzyme dihydro-orotate dehydrogenase (DHODH), a key enzyme in the de novo production of pyrimidines in T-lymphocytes, a process essential for T-lymphocyte proliferation.

Leflunomide showed antirheumatic activity which is comparable to methotrexate and sulphasalazine in randomized controlled trials using an oral dosage regimen starting with 100 mg once daily for three days followed by a maintenance dose of 20 mg once daily [2]. Overall in these trials 50–67% of patients reaches efficacy end points during 12 month follow up, with some patients reaching clinical efficacy 4–6 months after start of leflunomide therapy. Adverse events most frequently reported are gastrointestinal complaints (diarrhea, abdominal pain). The combination of late onset of efficacy, the high incidence of adverse events early in therapy and the uniform dosing schedule limit drug survival rates of leflunomide in populations with rheumatoid arthritis [3–5].

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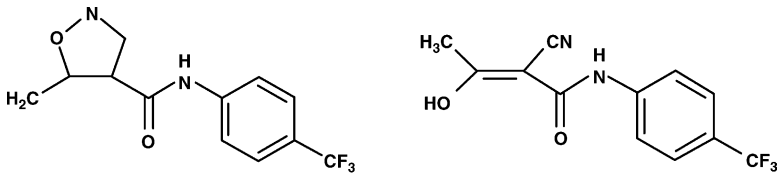

(A) (B)

Fig. 1. Chemical structures of leflunomide (A; CAS 75706-12-6) and its major metabolite A77 1726 (B; 108605-62-5).

On the basis of these results optimization of leflunomide therapy is needed.

An option for treatment optimization is dose adjustment on the basis of A77 1726 steady state serum concentrations. In preregistration pharmacokinetic population modeling studies, a relationship between steady state A77 1726 serum concentrations and the probability of clinical success is suggested [6]. Leflunomide is rapidly and completely metabolized after oral administration, for that reason serum concentrations are unmeasurable. Furthermore, no clinically relevant inhibition of DHODH by leflunomide is supposed, leaving the antirheumatic activity of leflunomide negligible. Therapeutic drug monitoring therefore should focus on the major active metabolite A77 1726.

Earlier, published methods for determination of A77 1726 had several drawbacks. For example these methods lack information on interference of detection and quantitation of the analyte, A77 1726, by co-medication frequently used in patients with rheumatoid arthritis [7]; did not consider hydrolysis of leflunomide in extraction solvents containing potassium carbonate [8]; or had a upper limit of quantitation (due to loss of linearity) that did not cover the complete concentration range expected to be obtained in patient samples [9,10]. Moreover, information on the stability of A77 1726 in serum kept under refrigerated conditions for more than one month [7] was lacking.

To study the potential value of therapeutic drug monitoring of A77 1726 in optimizing the treatment schedule, a validated method of analysis in human serum is needed. In this study we present a rapid and simple, validated HPLC-UV method for A77 1726 in human serum. Moreover, data are presented on the range of steady state serum concentrations of A77 1726 in a population of patients with rheumatoid arthritis on 10–20 mg leflunomide daily.

2. Material and methods

2.1. Equipment

Chromatographic separation was performed using a Waters High-performance liquid chromatography (HPLC) apparatus (Milford, MA, U.S.A.) consisting of a gradient pump and a column heater (model 2690) and a variable wavelength detector (model 996 PAD). Isocratic chromatographic separation was performed on a reversed-phase LiChrospher 100 RP-18e column (5 μm; 125 mm × 4 mm; Merck (Darmstadt, Germany)). The column temperature was maintained at 25 °C.

All samples and standard solutions were chromatographed using a mixture of methanol: KH_2PO_4 (45 mM, pH = 3) (50:50, v/v) as the mobile phase (flow rate 1.0 mL min^{-1}), and UV-detection at 295 nm and an injection volume of 20 μL. Data from each chromatographic run were processed using Waters Millennium 32 software. Concentrations were calculated from the peak height ratios in relation to the internal standard.

2.2. Preparation of the mobile phase

The mobile phase (methanol: KH_2PO_4 (45 mM, pH = 3) (50:50, v/v)) was prepared by adding methanol and the KH_2PO_4-buffer and mixing well. The KH_2PO_4-buffer (45 mM, pH = 3) was prepared by dissolving 6.124 g KH_2PO_4 in 1000 mL distilled water and correcting the pH to 3 with phosphoric acid 85%. The mobile phase was daily degassed and filtered before use.

2.3. Chemicals

Acetonitrile and methanol were purchased from Labscan Ltd (Dublin, Ireland). Ethanol, KH_2PO_4, demoxepam and phosphoric acid 85% were obtained from Merck (Darmstadt, Germany). Acetaminophen, azathioprine, celecoxib, diclofenac, hydrochloroquine, ibuprofen, methotrexate, naproxen, rofecoxib, prednisolone, sulphapyridine, sulphasalazine, 5-aminosalicylic acid, 6-mercaptopurine were purchased from Bufa (Uitgeest, The Netherlands). Leflunomide and A77 1726 were kindly provided by Aventis Pharma (Hoevelaken, The Netherlands). Human serum was derived from a pool of anonymous and unpaid, healthy volunteers.

2.4. Preparation of standard solutions and samples

For preparation of the stock standard solution, A77 1726 was dissolved in ethanol at a concentration of 1 mg/mL, placed in an ultrasonic water bath for 1 h and subsequently diluted to 100, 10 and 1 mg/L by spiking blanc human serum.

For the stock internal standard solution, demoxepam was dissolved at a concentration of 2.5 mg/L in acetonitrile.

Patient serum samples were prepared by adding 1.0 mL internal standard solution to 100 μL serum. The tubes

were capped, vortexed for 3 s and centrifuged at 3000 rpm for 5 min. Two-hundred microlitre of the supernatant was transferred into a glass tube and evaporated to dryness under a gentle nitrogen stream at 40 °C. The residue was dissolved in 200 μL of the mobile phase. The reconstituted specimens were vortexed for 10 s and analysed with HPLC-UV.

Serum calibration standards (0.5, 1, 10, 25, 50, and 100 mg/L) and quality control standards were separately prepared by spiking blanc pooled human serum with increasing amounts of A77 1726 stock standard solution and further processed as patient serum samples.

2.5. Validation

Linearity was examined over the complete concentration range of A77 1726 (0.5–100 mg/L) and using acceptable fit to linear regression by calculating regression coefficients and evaluation of sum of squares of residuals, tested with analysis of variance.

Selectivity was examined by studying the interference of endogenous peaks and antirheumatic medication with the determination of A77 1726 and the internal standard. Criteria set for lack of interference were a resolution between the peak of A77 1726/internal standard and potentially interfering medication of >2. Selection of potentially interfering antirheumatic drugs was based on UV-absorption spectra at 295 nm. These drugs were studied at serum concentrations reached with doses routinely used for rheumatoid arthritis. Drugs and their major metabolites studied were non-steroidal anti-inflammatory drugs (celecoxib, diclofenac, ibuprofen, naproxen, rofecoxib), disease-modifying antirheumatic drugs (DMARDs; azathioprine/6-mercaptopurine, hydrochloroquine, methotrexate, sulphapyridine, and sulphasalazine/5-aminosalicylic acid) and acetaminophen. Since (methyl) prednisolone and prednisone do not have relevant absorption of UV at 295 nm, these compounds are very unlikely to interfere with detection and quantitation of A77 1726. However, to confirm this, the major metabolite of prednisone, prednisolone, was studied for interference.

The intra-day reproducibility was examined by analysing five independent preparations of each standard concentration, each injected twice, on the same day. Inter-day reproducibility was examined by analysing five independent preparations of each concentration, each injected twice, on three different days within a period of 2 weeks. For each day freshly prepared standard solutions were made. All standard solutions were prepared from an independent standard stock solution. The calibration curve used for calculating patient samples was the calibration curve determined from all data of the five separate repetitions of intra-day reproducibility testing. These data were fitted using least sum of squares analysis. Criteria for acceptance for each separate calibration curve were a regression coefficient of >0.99 and a back-calculated concentration within 15% of the respective target concentrations.

Stability of A77 1726 40 mg/L in human serum was studied by repeated, duplicate analysis. The solution was divided into 5 mL aliquots and kept frozen at −20 °C until the moment of analysis. Samples were analysed at 0, 1, 2, 4 weeks and every 4 weeks for a period of 5 months, subsequently. Criteria for acceptable stability were differences between the baseline concentration and the concentration at follow up <5%.

2.6. Patient samples

All consecutive patients visiting our outpatient department of rheumatology from January to October 2003, who were on stable, daily doses of leflunomide for at least 6 months were asked to participate in this part of the study. After obtaining written informed consent a single venous blood sample in anti-coagulant-free evacuated containers was taken for determination of A77 1726. After sampling, blood was directly centrifuged and serum was kept frozen at −20 °C. Directly prior to analysis samples were processed as described above. In case of serum concentrations of A77 1726 above the upper limit of quantification, samples were diluted 1:1 with blanc human serum and re-analysed. All patient samples were injected twice, comparable to standard and control samples. The Medical Ethical Committee approved the study.

3. Results

3.1. Method validation

With the described method resolution >2 of A77 1726 and the internal standard (demoxepam) was achieved (Fig. 2). The retention times of demoxepam and A77 1726 are 5.8 and 8.9 min, respectively.

Chromatograms displayed no interference of endogenous peaks in spiked blanc human serum samples and patient samples. No interference of antirheumatic medication with peaks of demoxepam and leflunomide was detected, with all co-medication peaks having a resolution >2 compared to the peaks of A77 1726 or the internal standard.

The calibration curve for A77 1726 was linear over the full concentration range from 0.5 to 100 mg/L, with a correlation coefficient of 0.9996. Correlation between the ratio as calculated from the chromatograms and the spiked concentrations is best described by:

$$[\text{Concentration } A77\,1726]\,(\text{mg/L}) = 11.5 \times [\text{peak height ratio}] + 0.14$$

Table 1 shows the results of the intra- and inter-day reproducibility, respectively. Coefficients of variation and

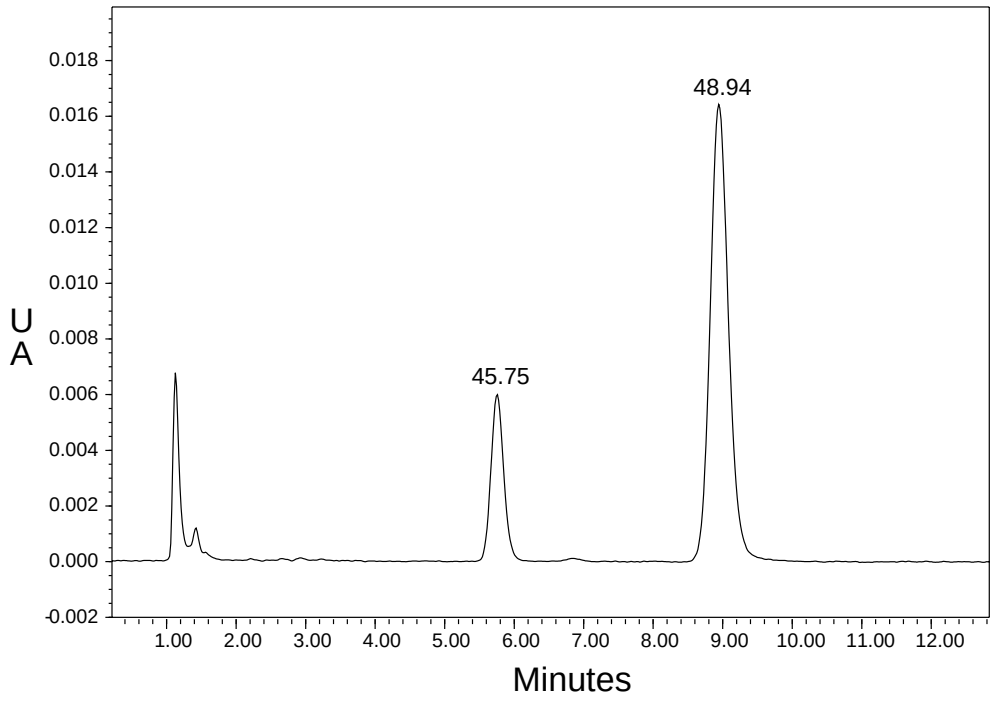

Fig. 2. Chromatogram of serum of a patient with a A77 1726 serum concentration of 32 mg/L (retention times of A77 126 and demoxepam (internal standard), 8.9 and 5.8 min, respectively.

Table 1
Intra- and inter-day reproducibility for A77 1726 in human serum

Spiked (mg/L)	A77 1726 Intra-day reproducibility[a]			A77 inter-day reproducibility[b]		
	Measured (mg/L)	CV (%)	Accuracy (%)	Measured (mg/L)	CV (%) (mg/L)	Accuracy (%)
0.5	0.52 ± 0.03	8.1	4.4	0.53 ± 0.03	5.0	7.0
1	1.08 ± 0.03	2.8	8.0	0.94 ± 0.12	13.2	−5.9
10	9.95 ± 0.06	0.6	−0.5	10.03 ± 0.30	3.0	0.3
25	24.5 ± 0.05	0.2	−1.9	24.6 ± 0.52	2.1	−1.8
50	48.0 ± 0.03	0.1	−3.9	48.4 ± 0.73	1.5	−3.2
100	100.2 ± 0.15	0.1	0.2	100.7 ± 0.61	0.6	0.7

CV = coefficient of variation.
[a] From 5 repetitions each.
[b] Three separate days, 5 repetitions each day.

accuracy for intra- and inter-day reproducibility are within 15% over the concentration range from 0.5 to 100 mg/L. On the basis of these results, the lower and upper limits of quantitation for A77 1726 with this analytical method are 0.5 and 100 mg/L respectively.

Results from the assays for studying stability of A77 1726 over the period of 5 months are shown in Table 2. Until week 8 samples show acceptable stability, with differences between the baseline concentration and concentration at follow up <5%.

Table 2
Stability of A77 1726 40 mg/L in human serum

T (weeks)	Measured concentration (mg/L; $n = 2$ each)	Deviation from $T = 0$ (mg/L)	Devation from $T = 0$ (%)
0	40.7	–	–
1	41.7	1.0	2.4
2	42.6	1.9	4.7
4	42.1	1.4	3,4
8	40.9	0.2	0,5
13	43.9	3.2	7,9
17	41.3	0.6	1,5
22	38.2	−2.5	−6.1

Table 3
Patients characteristics for population included for determination of A77 1726 concentrations

Characteristics	
Number of patients	37
Age (years)	70 [12]
Duration of RA (years)	12 [10]
Rheumatoid factor positive (%)	76
Leflunomide prescribed as first DMARD (%)	16
Number of DMARDs prior to leflunomide	2.0 (1.6)
Duration of leflunomide use (days)	970 (237)
Range	214–1281
Prescibed daily leflunomide dose (mg)	
10	8
15 (alternating 10 and 20 mg)	2
20	27

Data are mean (S.D.) unless stated otherwise; DMARD = disease modifying antirheumatic drug; RA = rheumatoid arthritis.

3.2. Patient samples

Thirty-seven blood samples were taken for determination of A77 1726 concentrations. Patient and treatment characteristics of the population are given in Table 3. Measured serum concentrations show large variability, ranging from 3 to 176 mg/L. For two patients (5%) A77 1726 serum concentrations were found to be >100, 133 and 176 mg/L, respectively. Mean (S.D.) concentrations for the 10 and 20 mg daily dose groups are 39 (30) and 42 (37) mg/L, respectively.

4. Discussion

The current analytical method shows acceptable intra- and inter-day accuracy and precision over the A77 1726 concentration range from 0.5–100 mg/L. Endogenous serum peaks and antirheumatic medication did not interfere with the detection of A77 1726 in the current HPLC-UV method. Stability of A77 1726 in human serum kept frozen at −20 °C until the moment of analysis is shown up till 8 weeks after sampling. This enables analysing patient samples in one run up to 8 weeks after sampling in future studies.

To study the applicability of the validated method in clinical practice a series of patient serum samples were analysed for A77 1726 concentrations. The serum concentrations are characterized by large interindividual variation. The validated concentration range covers 95% of the concentration found in the patient samples in our study.

As stated, previously published methods for determination of A77 1726 had several drawbacks concerning lack information on interference of detection and quantitation of the analyte, A77 1726, by antirheumatic co-medication [7]; did not consider hydrolysis of leflunomide in extraction solvents containing potassium carbonate [8]; or had an upper limit of quantitation (due to loss of linearity) that did not cover the complete concentration range expected to be obtained in patient samples [9,10]. Moreover, information on the stability of A77 1726 in serum kept under refrigerated conditions for more than one month [7] was lacking. The HPLC-UV method described here, focuses on A77 1726 as the only analyte of interest, is validated for the absence of interference of other, commonly prescribed antirheumatic medication on the determination of A77 1726 and studies stability of A77 1726 in serum for a period up till 5 months.

A77 1726 is the metabolite responsible for inhibition of DHODH and therefore antirheumatic activity. Moreover, reports on pharmacokinetics of leflunomide show that no detectable serum levels of leflunomide are reached in dose regimens for rheumatoid arthritis [1]. A77 1726 itself is excreted in faeces or metabolised further to 4-trifluoromethylaniline oxalinic acid [1]. The role of leflunomide and the metabolites of A77 1726 in antirheumatic activity are currently not clear and systemic exposure to these metabolites is minimal [1]. Therefore, quantitative assays for use in patient samples should focus on A77 1726 as the analyte of interest.

A proportion of the patients with rheumatoid arthritis will be treated with combinations of DMARDs, analgesics and/or corticosteroids, within the current treatment paradigm of rheumatoid arthritis. Therefore, interference of concomitant medication with the quantitation of A77 1726 is of special interest in the patient group treated with leflunomide. Our method is validated for absence of interference of concomitantly prescribed antirheumatic drugs and their main metabolites on the quantitation of A77 1726.

5. Conclusion

We developed an easy-to-operate and validated HPLC-UV method for determination of A77 1726, the active metabolite of leflunomide, in human serum. The proposed method can be employed for the assay of A77 1726 in rheumatoid arthritis patient samples.

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