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Determination of laquinimod in plasma by coupled-column liquid chromatography with ultraviolet absorbance detection

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Abstract

Laquinimod is an immunomodulator that is currently in clinical trials. For pharmacokinetic and toxicokinetic studies in animals and humans a sensitive and accurate bioanalytical method was required. In this paper a bioanalytical method for the determination of laquinimod by liquid chromatography is described. After a protein precipitation step the plasma sample was injected onto a coupled-column HPLC system. After further purification from macromolecules on a short restricted access material C_{18} column the analyte was transferred to a reversed-phase C_{18} analytical column and separated from interfering substances. The analyte was detected by UV detection. The method was validated with respect to linearity, selectivity, precision, accuracy, limit of quantitation, limit of detection, recovery and stability. The limit of quantitation was 0.75 μmol/L, the intermediate precision was 1.8–3.6% (C.V.) and the accuracy was 97.7–114.7%. In conclusion, the method was found to perform well and is suitable for use in pharmacokinetic and toxicokinetic studies.

Keywords: Coupled columns; Laquinimod

1. Introduction

Laquinimod, ABR-215062, 5-chloro-1,2-dihydro-*N*-ethyl-4-hydroxy-1-methyl-2-oxo-*N*-phenyl-3-quinolinecarboxamide (Fig. 1), is a synthetic compound which inhibits inflammatory disease activity (*Journal of Neuroimmunology*, in press). The compound originates from a project based on the preclinical and clinical knowledge generated using a quinoline-3-carboxamide derivative, roquinimex (Linomide, ABR-212616). Laquinimod has an improved therapeutic window compared with roquinimex (manuscript in preparation).

The bioanalytical method for the determination of laquinimod in plasma described here was developed and validated to enable pharmacokinetic and tox-

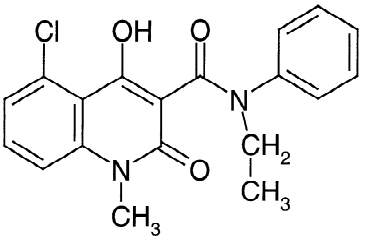

Fig. 1. The structure of laquinimod.

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icokinetic studies. In order to rationalise sample preparation, a column-switching (coupled-column) HPLC technique was chosen. Off-line sample preparation (SPE) methods often include a concentration step which considerably increases the total analysis time. Coupled-column chromatographic methods, on the other hand, give the same degree of sample purification, concentration and selectivity as SPE. Furthermore, they allow direct injection of plasma samples and are therefore considerably less labour-intensive. No concentration step is needed before injection [1–8]. A simple protein precipitation step was introduced, however, and increased the pre-column lifetime considerably.

2. Experimental

2.1. Materials

Laquinimod (see Fig. 1) was synthesised at the Active Biotech Research AB laboratories in Lund. Acetonitrile and tetrahydrofuran (Labscan, Dublin, Ireland) were of HPLC grade, (85%) phosphoric acid (Janssen Chimica, Geel, Belgium), (47%) potassium hydroxide (Merck, Darmstadt, Germany) and (25%) ammonia (BDH, Poole, UK) were of analytical grade. Water was deionized by a Milli-Q water purification system (Millipore, Bedford, MA, USA). Ultrafree-MC 0.45 μm filters were purchased from Millipore.

2.2. Instrumentation

The high-performance liquid chromatographic (HPLC) system consisted of a Waters 600E pump (Waters Chromatography Division, Millipore, Milford, MA, USA), a HP 1100 pump (Hewlett-Packard, Avondale, PA, USA), a Waters 717 plus autosampler, a Waters 486 UV detector, a Valco six-port coupling valve (VICI Valco Instruments, Houston, TX, USA) and a Waters Millennium 32 Chromatography Manager. The pre-column was a LiChroCART LiChrospher RP-18 ADS, 25 μm, 25×4 mm I.D. (Merck). The stationary phase type was RAM (restricted access material, C_{18}), consisting of spherical porous particles with a hydrophilic and electroneutral outer surface which repels macromolecules, and pores with inner walls coated with hydrophobic C_{18} chains with reversed-phase properties. The reversed-phase analytical column was a Zorbax SB-C_{18}, 5 μm, 150× 4.6 mm I.D. (Rockland Technologies, Neunen, Netherlands). An Opti-guard C_{18}, 15×1 mm I.D. (Optimize Technologies, Oregon City, OR, USA) was used as guard column.

2.3. Chromatographic conditions

The pump serving the pre-column operated at 1.0 mL/min and the pump serving the analytical column operated at 0.7 mL/min. The pre-column mobile phase was 0.01 *M* phosphate buffer, pH 3.2, and the analytical mobile phase was 0.01 *M* phosphate buffer, pH 3.2–acetonitrile–tetrahydrofuran (50:45:5). The autosampler temperature was set at +8 °C and the column was maintained at ambient temperature (22–24 °C). The injection volume was 50 μL. UV absorption was monitored at a wavelength of 330 nm. The column switch valve was controlled by Millennium software and operated as follows: separation of analyte and matrix on pre-column (0–2.5 min), transfer of analyte onto the analytical column (2.5–5.5 min) and pre-column conditioning (5.5–12.0 min).

2.4. Sample preparation

Plasma was thawed, centrifuged and 0.2 mL of the clear liquid was mixed with 100 μL of 1 *M* phosphoric acid. The mixture was filtered by centrifugation through an Ultrafree-MC 0.45 μm filter for 2 min at ≥12 000 *g*. The supernatant was transferred to a HPLC vial, and 50 μL was injected onto the two-column HPLC system.

The plasma macromolecules were allowed to pass through the RAM column, while the analyte and other small molecules were adsorbed. The flow of the mobile phase was then reversed and the analyte was transferred to the analytical column by a mobile phase with stronger eluting power and then separated from interfering substances.

2.5. Calibration

Calibration standards were included in every analytical batch of samples. The calibration stan-

dards were prepared by spiking blank plasma at seven different concentrations in the range 0.75–967 μmol/L. The standards were processed as described above for the samples. Calibration curves were calculated by the Millennium chromatography manager, which also controlled the HPLC system. The peak areas of laquinimod were plotted versus the concentration in the calibration standards.

The method of external standard calibration was applied and a linear, weighted ($1/C^2$, where C is the concentration) calibration curve was calculated from the peak areas of the standards. A $1/C^2$ weighting factor was selected because it gave the best curve fit at low concentrations compared with the available alternatives (no weighting, C, C^2, $\log C$, $1/C$ and $1/C^2$).

3. Results

3.1. Selectivity

Laquinimod gave a well-defined peak with the chromatographic system used (Fig. 2b). Drug-free plasma was tested for interference from endogenous components, and no interfering peaks were detected. Chromatograms obtained for blank plasma and plasma spiked with laquinimod (1.1 μmol/L) are shown in Fig. 2a and b, respectively. The small peak present at the retention time of laquinimod in the blank plasma chromatogram is well below the calculated limit of detection. Diode-array detection showed that the substance peak was pure (the spectra over the peak were consistent) and extension of the chromatogram over 60 min did not reveal any new peaks.

3.2. Linearity

The calibration graph over the range 0.75–967 μmol/L was linear and the correlation coefficient (r) was ≥0.995. The coefficient of variation (C.V.) ranged from 0.7 to 6.4% and the largest deviation was −11%.

3.3. Precision and accuracy

Human plasma was spiked with laquinimod at three different concentrations: 1.99, 207 and 829 μmol/L. Aliquots of 0.2 mL were deep frozen and stored at −70 °C. Four replicates of the plasma samples were analysed on six different occasions.

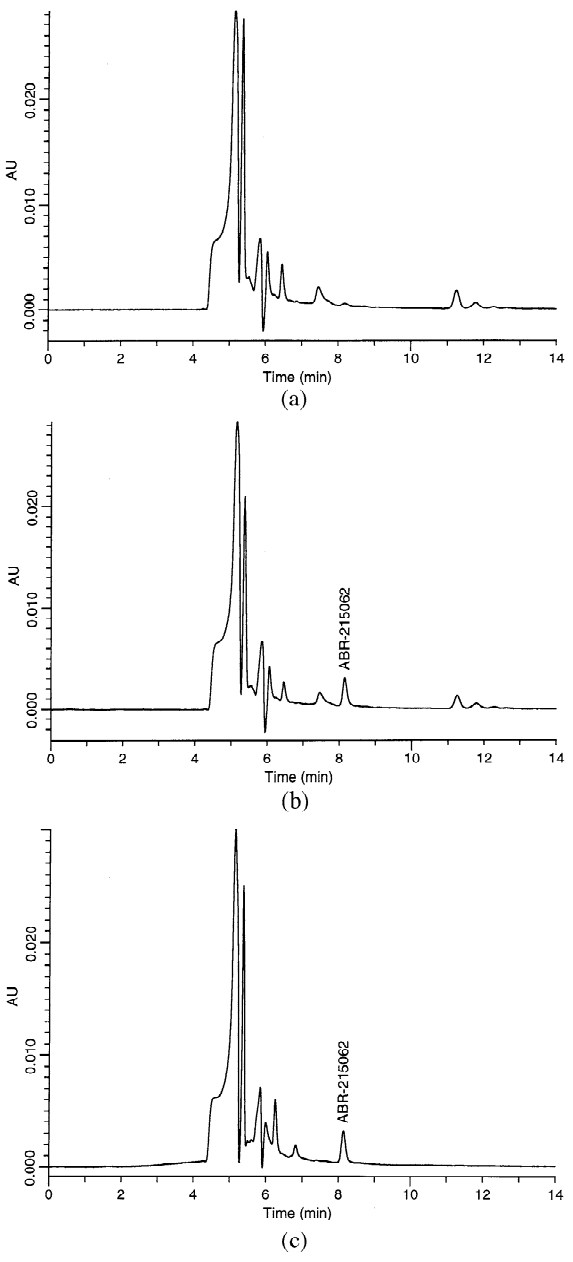

Fig. 2. (a) Chromatogram of blank plasma. (b) Chromatogram of blank plasma spiked with 1.1 μmol/L laquinimod. (c) Chromatogram of rat plasma 24 h after oral administration of 10 mg/kg laquinimod (1.02 μmol/L).

The repeatability (intra-assay precision), defined as the coefficient of variation, C.V. (where the standard deviation was calculated as

$$S = \sqrt{\sum(n_i \times S_i^2)/\sum n_i}$$

with n_i and S_i being the number of samples analysed and the standard deviation of day i), ranged from 0.5 to 1.1%. The intermediate (inter-assay) precision, defined as the C.V., ranged from 1.8 to 2.5%. The accuracy was in the range 97.7–102.6%. The results are summarised in Table 1.

3.4. Limit of detection

To estimate the limit of detection (LOD), 0.2 mL of blank plasma was analysed in triplicate on eight different occasions. The apparent concentrations detected at the retention time of the analytes were calculated. The LOD was defined as the mean $+ t \times$ SD, where SD is the standard deviation of the apparent concentrations obtained in blank plasma and t the t-distribution at the 95% level. The limit of detection was determined to be 0.4 μmol/L.

3.5. Limit of quantitation

Human plasma was spiked with laquinimod at 0.75 μmol/L. Aliquots of 0.2 mL were deep frozen and stored at −70 °C. Six replicates of the plasma samples were analysed on two different occasions. The repeatability was 1.6%, the intermediate precision 3.6% and the accuracy 114.7%. The results are presented in Table 1.

The limit of quantitation (LOQ) was defined as the lowest concentration with a precision <20%, an accuracy of 80–120% and with a signal that significantly (95%) differed from the signal at the limit of detection. The LOQ concentration was 0.75 μmol/L (Table 1).

3.6. Recovery

The recovery was determined by spiking plasma samples with laquinimod at 2.1 and 244 μmol/L, and then processing and injecting in accordance with the method. As references, solutions of laquinimod in mobile phase of the corresponding volumes and concentrations were injected directly onto the analytical column (the pre-column was removed from the system). The peak areas of laquinimod in the prepared samples were compared with the peak areas of reference solutions of laquinimod. The recovery of the assay was close to 100%.

3.7. Stability

Prepared samples are stable during storage in the autosampler (+8 °C) for at least 46 h.

Blank plasma from human, rat, dog and mouse was spiked with laquinimod at 2, 100 and 400 μmol/L, respectively. The samples were stored at −20 °C. Triplicate samples were analysed within 2 days of preparation and after 2, 3, 4 and 6 months. Samples spiked into human plasma were also assayed after 12 months.

A maximum concentration change of 10% was accepted to ensure that the accuracy of the samples remained within 85–115%. The regression line of plasma concentration found versus storage time was calculated.

Laquinimod was stable in animal plasma samples

Table 1
Precision and accuracy of the method

Nominal conc. (μmol/L)	Conc. found (mean) (μmol/L)	Repeatability (C.V., %)	Intermediate precision (C.V., %)	Accuracy (%)	Number (n)
0.75	0.86	1.6	3.6	114.7	12
1.99	1.94	1.1	1.9	97.7	24
207	213	0.5	1.8	102.6	24
829	845	0.8	2.5	101.9	24

for at least 6 months when stored at −20 °C, with only minor compound degradation being observed during this period. In human plasma, an increase in concentration of about 12% was calculated for the 2 μmol/L samples after 12 months. A maximum storage time of 10 months for human plasma samples was derived from the regression line.

The plasma samples were also found to be stable for at least three freeze–thaw cycles, and at room temperature for at least 2 h.

3.8. Applications

The method was used for the determination of laquinimod in plasma samples from preclinical studies. The precision and accuracy derived from quality control samples, co-analysed with the study samples, were in accordance with the values achieved during the validation. The sensitivity of the method has been adequate for the toxicokinetic evaluations performed in the safety studies so far, where doses of 0.3–90 mg/kg have been studied.

A chromatogram of a rat plasma sample is shown in Fig. 2c, and a typical plasma profile of laquinimod in rat plasma is shown in Fig. 3. The raw data originate from a 4-week oral (gavage) toxicity study in rats. The curves are the calculated means of 10 animals/sex/dose. The C_{max} and AUC values were slightly lower than expected at the highest dose, indicating non-linear kinetics.

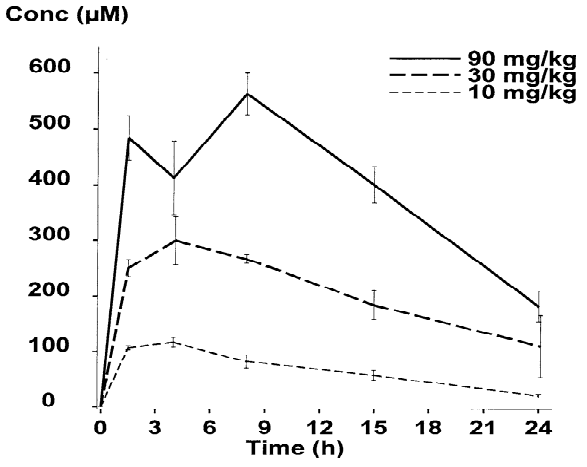

Fig. 3. Plasma concentration (mean±SEM)–time profile after a single oral administration of 10, 30 or 90 mg of ABR-215052 to rats.

4. Discussion

The method presented in this paper has proven to be robust. Direct injection of plasma after a simple protein precipitation step has proved to give a simple and accurate assay. The method was optimised with respect to sample throughput and therefore the sample preparation time was minimised. As there was a risk that the short sample preparation time could impose a strain on the columns due to precipitation of macromolecules, a protein precipitation step was included in the method. With the protein precipitation step implemented, the life span for the pre-column was 400–500 injections and at least 3000 injections for the analytical column. The sample throughput is high relative to manual input due to the high degree of automation, and the full capacity of the autosampler (96 positions) can be used.

A low pH (3.2) of the mobile phase was chosen to maintain the compound (pK_a 4.2) mainly in the non-ionised form to ensure sufficient retention on the column. A wavelength of 330 nm was chosen to achieve high selectivity against endogenous compounds in the chromatograms. At 330 nm the compound in the non-ionised form has a local absorbance maximum where the extinction coefficient is about one-third of the value at 254 nm.

The limit of quantitation was 0.75 μmol/L, the intermediate precision 1.8–3.6% (C.V.) and the accuracy 97.7–114.7%. Laquinimod was stable in plasma samples for at least 6 months when stored at −20 °C. The plasma samples were also found to be stable for at least three freeze–thaw cycles, and at room temperature for at least 2 h.

In conclusion, the procedure described provided good linearity, selectivity, precision and accuracy for the determination of laquinimod in plasma. The method is therefore suitable for pharmacokinetic and toxicokinetic studies of the compound. During preclinical safety studies, plasma samples from animals dosed with 0.3–90 mg/kg of laquinimod were analysed and the method was found to perform well.

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