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Quantitative determination of azithromycin in plasma, blood and isolated neutrophils by liquid chromatography using pre-column derivatization with 9-fluorenylmethyloxycarbonyl-chloride and fluorescence detection

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Abstract

In this study, a high-performance liquid chromatographic method with pre-column derivatization and fluorescence detection was optimised and validated for the quantification of azithromycin (AZM) in plasma. Clarithromycin (CLM) was used as an internal standard. Pre-column derivatization was done with 9-fluorenylmethyloxycarbonyl-chloride. Recovery from blood and polymorphonuclear neutrophils (PMNNs) isolated by a gravity separation procedure was also assessed. Analytical separation was carried out using a C18 column as stationary phase and acetonitril–phosphatebuffer as mobile phase. Peak quantification was carried out by excitation at 267 nm and detection at 317 nm. A lower limit of quantitation of 0.042 ± 0.017 mg/l in plasma, 0.119 ± 0.065 mg/l in blood and 0.072 ± 0.036 in water was achieved. Linearity was assessed from 0 to 1.5 mg/l in plasma and blood and from 0–9 mg/l in water. The analytical method proved to be applicable in a pharmacokinetic study of AZM in a Cystic Fibrosis patient.

Keywords: Azithromycin; Derivatization; LC

1. Introduction

Azithromycin (AZM) is a 15-membered ring azalide antibiotic related to the macrolide antibiotics. AZM is active against a wide number of Gram-positive bacterial pathogens like *Streptococci*, *Staphylococcus aureus*, *Propionibacterium acnes* and *Listeria monocytogenes*. Gram-negative bacteria like *Haemophilus influenzae*, *Moraxella catarrhalis*, *Legionella pneumonia* and *Neisseria gonorrhoeae* are also susceptible to AZM. AZM is also active against bacteria that cause intracellular infections like *Mycobacterium avium* and *Chlamidia trachomatis* [1].

AZM exhibits a bacteriostatic effect on susceptible pathogens by interfering with RNA-dependent protein synthesis [2].

AZM is widely used for upper and lower respiratory tract infections, infections of skin and soft tissue and sexually transmittable infections of bacterial origin.

AZM is administered for a short period of time (usually 3 consecutive days) [1]. Accumulation of AZM in tissue and white blood cells, both polymorphonuclear neutrophils (PMNNs) and monocytes, has been associated with a prolonged antimicrobial effect [3,4].

AZM is able to reduce or stabilize clinical symptoms of airway inflammation of patients chronically infected with *Pseudomonas aeruginosa*. A chronic infection with *Pseudomonas aeruginosa* often occurs in patients with diffuse panbronchiolitis and cystic fibrosis [5,6,7]. Although the min-

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imal inhibitory concentration (MIC) for *Pseudomonas aeruginosa* is high (up to 128 mg/l) in vitro data suggest that long-term exposure to sub-MIC concentrations are bactericidal [8]. To date, the exact mechanism and the optimal dosing schedule for chronic administration of AZM in pulmonary infections with *Pseudomonas aeruginosa* remains unclear.

No pharmacokinetic investigations have been carried out so far to support chronic dosing in these pathologies. Investigations after short-term administration (3 days) showed accumulation of AZM in white blood cells [4]. However, the amount of intracellular accumulation at chronic dosing is unknown. In order to quantify the amount of accumulation in PMNNs, we adapted a separation technique to select PMNNs by gravity separation [4]. For further pharmacokinetic studies the analytical method has to be suitable for quantification of AZM in blood, plasma and isolated PMNNs.

Lack of UV absorption of macrolide antibiotics makes that fluorescence detection can only be used when macrolides are coupled to a fluorescent compound. Otherwise, an alternative detection system, like electrochemical detection or mass spectrometry has to be applied [9,11–14]. We modified the method described by Sastre Torano and Guchelaar who used high-performance liquid chromatography with pre-column derivatization with 9-fluorenylmethyloxycarbonyl-chloride (FMOC-Cl) and fluorescence detection [10]. This method has also been used for the quantification of macrolide antibiotics in foods. Sastre Torano and Guchelaar validated this method to quantify four macrolide antibiotics (erythromycin, roxitromyin, AZM and clarithromycin) in serum within the same HPLC run. The lower limit of quantification of the described method was 0.09 mg/l for AZM and 0.2 mg/l for clarithromycin (CLM) in a 1 ml sample [10].

We optimised this method to be able to quantify AZM with a lower limit of quantification (LOQ) of 0.05 mg/l in a 0.5 ml sample and validated this method for plasma using CLM as an internal standard. We also determined the recovery, intra-assay variation and LOQ in blood and isolated PMNNs.

2. Experimental

2.1. Materials

All chemicals and solvents were of analytical or HPLC grade and were used without further purification. AZM dihydrate salt (Pfizer Inc., New York, USA) and CLM (Abbott, Queenborough, UK) were used for spiking blanks. 9-fluorenylmethoxycarbonyl-chloride (Sigma–Aldrich, Zwijndrecht, The Netherlands) was used for derivatization.

Methanol (Lichrosolv), acetonitrile (Lichrosolv), potassium dihydrogen phosphate, di-sodiumcarbonate anhydrous, diethyl ether and potassium hydroxide were purchased from Merck (Darmstadt, Germany).

Ficoll-Paque Plus (Amersham Biosciences, Uppsala Sweden) gravity separation medium, phosphate buffered saline (PBS) pH 7.4 (Mallinckrodt-Baker, Deventer, The Netherlands), sodium chloride 0.9% for irrigation and purified water for irrigation (both from Baxter Inc. Utrecht, The Netherlands) were used in the neutrophil separation procedure.

Flow cytometric analysis of isolated cell fraction was performed with anti-cd15 fluorescence probe (BD Biosciences, San Jose, USA).

2.2. Instrumentation and chromatographic conditions

Chromatographic conditions were optimised towards the quantification of AZM with CLM as internal standard, using the settings published by Sastre Torano and Guchelaar as a start. The concentration of acetonitril in the mobile phase, the column and flow rate were varied to optimise the quantification.

We used an Agilent HPLC system (Agilent 1100 series) which consisted of a Symmetry C18 column (4.6 mm × 100 mm, 3.5 μm; Waters WAT066265), and a Symmetry C18 pre-column (4.6 mm × 20 mm, Waters WAT054225)), a solvent delivery pump and degasser, an auto-injector, a column thermostat, and a fluorometric detector (λ excitation −267 nm, λ emission 317 nm; peak width >0.2 min., photo multiplier gain 10) and a diode array detector (DAD). Chemstation integrating software was used (Agilent Inc., Palo Alto, Ca, USA).

The mobile phase we used in the validation process consisted of a mixture of 760 ml acetonitril and 240 ml 0.02 M phosphate buffer (pH 7.7). The phosphate buffer contained 0.65 g potassium dihydrogen phosphate in 240 ml distilled water adjusted to pH 7.7 with potassium hydroxide 10% in distilled water.

An injection volume of 50 μl, a flow rate of 1.5 ml/min, a pressure of 115 bar and a column temperature of 28 °C were applied.

Differentiating cell counts were made with a Coulter Counter type Onyx (Beckman Coulter Inc., Pasadena, Ca, USA). Cell identification was performed with a flowcytometer FACS Calibur (BD Biosciences, San Jose, USA).

2.3. Isolation procedure of PMNNs

PMNNs were isolated from 6 ml lithium-heparin venous blood samples. After a differentiating count of blood cells, 6 ml blood was diluted with 6 ml PBS. This was transferred on a 6 ml layer of Ficoll-Paque density separation medium and centrifuged for 15 min (1250 *g*) at 21 °C. The supernatant and Ficoll-Paque layer were removed and the cell pellet with PMNNs and erythrocytes was incubated 15 min with 45 ml of NaCl 0.2% at 2–6 °C in order to lysate erythrocytes. PMNNs were isolated by centrifugation (5 min, 465 *g*) and resuspended in 6 ml PBS at room temperature. A cell-differentiating count was performed to determine the number of isolated PMNNs. After centrifugation (5 min, 465 *g*) the supernatant was removed and the cell pellet was kept at −30 °C until determination of AZM.

2.4. Sample treatment

Plasma and blood were obtained from lithium-heparin blood sampling tubes and kept at −30 °C until determination. Quantification of the amount of AZM in PMNNs took place after thawing and addition of water until a volume of 1.2 ml.

2.5. Preparation of a calibration curve

A methanolic AZM stock solution (about 10 mg/50 ml) was diluted to a work solution of 0.50 mg/50 ml methanol. A calibration curve of 0.1–1.0 mg/l was made by adding 5, 10, 15, 25 and 50 μl of the work solution to 0.50 ml of blank plasma. Additional methanol was added to the standards, so, each sample contained 50 μl of methanol.

For the determination of AZM in blood, a standard curve in blank blood samples was made. For the determination of AZM in isolated neutrophils, a standard curve in water was made.

Standard curves were constructed by non-weighed linear regression.

2.6. Sample extraction procedure

To 0.5 ml samples of plasma, blood and PMNN suspension a volume of 50 μl methanol was added. To the calibration curve and samples 100 μl of the internal standard CLM (1.0 μg/100 μl water) and 200 μl of a saturated disodiumcarbonate (0.4 mg/ml water, with a pH of approximately 11) were added in a 12 ml disposable glass tube and the solution was vortex-mixed for 2 s. A volume of 6 ml of diethyl ether was added to each tube. The tubes were shaken (200/min) for 15 min and centrifuged for 10 min (2550 *g*).

The organic layer was transferred into a disposable 12 ml glass tube and evaporated at 40 °C under a flow of N_2.

2.7. Derivatization procedure

In order to optimise the derivatization procedure we varied the amount and concentration of FMOC-Cl and the water–acetonitril ratio. The following derivatization procedure was found to be optimal: The residue from the organic layer was dissolved in 100 μl of acetonitril and vortex-mixed for 20 s. The solution was centrifuged for 10 s (2550 *g*) and quantitatively transferred into a 2 ml reaction vial. A 100 μl of a freshly prepared FMOC-Cl solution (1 mg/ml in acetonitril), 100 μl water and 75 μl phosphate buffer (0.1 M phosphate, adjusted to a pH of 7.5 with 10% potassium hydroxide) were added.

The reaction vial was incubated in a water bath at 40 °C for 40 min. After derivatization 50 μl of the solution was injected directly from the reaction vial into the HPLC system.

2.8. Assay validation

The selectivity of the method was investigated for a number of drugs commonly used by patients with cystic fibrosis. Prednisolone (1 mg/l), ceftazidim (200 mg/l), omeprazole (1 mg/l), ibuprofen (30 mg/l), sulfamethoxazole (60 mg/l), trimethoprim (2 mg/l), itraconazole (2 mg/l) and fluconazole (6 mg/l) were investigated for interference with the assay.

Precision of the HPLC system, intra-assay variation, inter-assay variation, linearity, recovery, the lower limit of quantitation (LOQ), accuracy and the stability were determined as validation parameters of the analytical method for the assay of AZM in plasma.

LOQ was calculated from the calibration curve constructed by non-weighed linear regression. We defined LOQ as the *y*-axis intercept plus 3.3 times the standard deviation and extrapolated this value towards *x*. In case, the intercept was negative, we defined LOQ as 10 times the standard deviation.

Recovery was assessed with a 0.5 ml plasma and blood. Recovery was also assessed from a blank PMNN suspension isolated from 6 ml Li-heparin blood sample spiked with AZM after the isolation procedure. After the extraction procedure CLM was added as internal standard. All extraction steps were performed quantitatively. A methanolic solution of AZM with the same concentration served as reference. Recovery was defined as the percentage of the concentration in the methanolic solution determined in the sample.

Stability was assessed in derivatized extracts, stock solution and working solution kept at −20 °C, plasma, blood and PMNN samples kept at −30 °C. The stock solutions and working solutions were compared with freshly prepared solutions. The plasma, blood and PMNN samples were assayed with an appropriate time interval and compared to the initial results. Samples were defined as stable when the results were within 3 × the sd.

2.9. Validation of neutrophil separation procedure

Recovery from a blank PMNN suspension was assessed as described above.

Recovery and intra-assay variation of the PMNN separation procedure was assessed in blood incubated with a known amount of AZM and in samples from two patients using AZM. AZM was quantified in PMNNs and in all other separation fractions and washing fluids. To 6 × 6 ml blank heparinised blood, 140 μg AZM was added (3.89 μg/ml). Blood was incubated and gently shaken during 2 h at 37 °C. The incubated blood samples were pooled together and homogenised. The pool was split into six samples of 6 ml. Each sample was treated according to the PMNN separation procedure. All washing fluids and the separation medium were harvested separately and frozen at −30 °C until assay. A mass balance was made after determination of the concentrations and the volumes of the samples. Purity of the cell selection was determined in the PMNN containing cell suspension by

flow cytometric analysis after labelling with an anti-cd15 fluorescence probe.

From two patients, 4 × 6 ml Li-heparin blood each were separated as described in the isolation procedure of PMNNs. AZM was determined in plasma and blood and in isolated PMNNs and in all washing fluids and in the separation medium in four-fold. A mass balance of all fractions was made and compared to the concentration found in blood and plasma. The intra-assay variation was determined.

2.10. Pharmacokinetic study

A pharmacokinetic study was conducted in a patient with cystic fibrosis who was treated with AZM 500 mg per day for at least 35 days. The study was approved by the medical ethical review board and written informed consent was obtained. Lithium-heparin sample tubes were used to obtain 2 × 6 ml venous blood per sample time. From 6 ml venous blood neutrophils were isolated. Samples were taken at $t = 0$, 1, 2, 3, 4, 6, 8, 74 and 220 h. AZM was determined in blood, plasma and PMNNs. The amount in PMNNs was expressed in amount of AZM in PMNNs per 1 l blood.

3. Results and discussion

3.1. Optimising chromatographic conditions

We increased the concentration of acetonitril in the mobile phase, which resulted in a decrease in retention time. We used a C18 column packed with 3.5 μm particles instead of 5 μm particles in a shorter column and with a reduced flow. This resulted in a reduction in retention time and in a better signal-to-noise ratio leading to a lower detection limit.

3.2. Optimising derivatization procedure

Variation of pH: An increase of the pH (>7.5) resulted in an increase in interferences. A reduction of the pH (<7.5) resulted in a reduction in AZM peak height. We, therefore, used a pH 7.5.

Variation of the acetonitril–water ratio: The acetonitril–water ratio was varied from 300–75 to 100–175. An increase of the water content led to a smaller chromatographic front without interference with the strength of the AZM signal.

Variation of the concentration of FMOC-Cl: The concentration of FMOC-Cl was varied from 0.25 to 2.5 mg/l. An increase in the concentration of FMOC-Cl to 2.5 mg/l led to a wider front, more interferences and a small increase in AZM signal. Reduction to 0.25 mg/l led to better chromatographic separation (smaller front and less interferences) and a small reduction in AZM signal, which was equal to the reduction in CLM signal. We found an optimum at a concentration of 1 mg/l and an added volume of 100 μl.

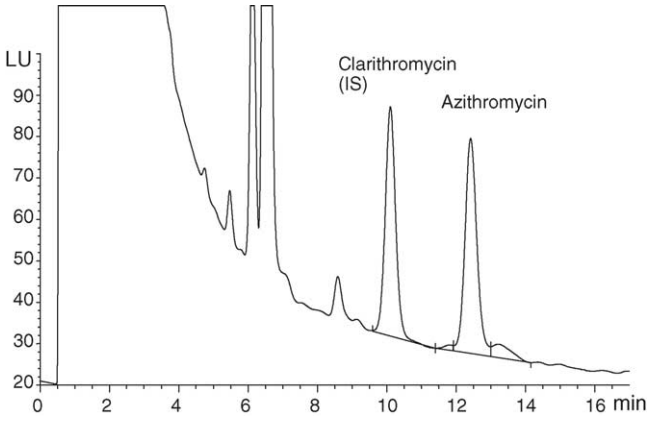

Fig. 1. A chromatogram of an extract from plasma spiked with AZM at a concentration of 0.74 mg/l and clarithromycin (internal standard) at a concentration of 1.0 mg/l.

We used a reaction time of 40 min. and a reaction temperature of 40 °C, which proved optimal in the method described by Sastre Torano and Guchelaar [10].

3.3. Selectivity

A representative chromatogram of AZM at 0.74 mg/l in plasma is shown in Fig. 1. The components prednisolone, ceftazidim, omeprazol, sulfamethoxazol, trimethoprim, itraconazol and fluconazol showed no interference with AZM and CLM. Ibuprofen (30 mg/l) showed some interference with CLM when detected with fluorescence detection. Detection with diode array showed no interference. Combined use of fluorescence detection for AZM and diode array detection for CLM can be used as an alternative in patients on high dosages of ibuprofen without loss of sensitivity. There were no interferences present in blank human plasma and isolated PMNNs.

3.4. Precision of the HPLC system

An organic solution of 0.3 mg/l AZM was injected six times. The coefficient of variation (vc) was 0.2%.

3.5. Intra-assay variation

Two spiked concentrations of AZM within the range of the calibration curve in plasma (0.317 mg/l and 0.634 mg/l) were assayed (each $n = 6$) on the same day in the same run. The coefficients of variation were 0.4 and 1.2%, respectively. A spiked sample (0.063 mg/l, $n = 6$) between the lowest sample of the calibration line and the LOQ was assayed with an intra-assay variation of 4.5%.

3.6. Inter-assay variation

Samples, containing AZM (0.635 mg/l, $n = 6$ and 1.182 mg/l, $n = 5$) in plasma were assayed on different days. The coefficient of variation was 2.2 and 2.8%, respectively.

In blood, the inter-assay variation of samples, containing AZM (0.295 and 0.739 mg/l) was 2.4 and 3.9%, respectively.

3.7. Linearity

The calibration curve of AZM resulted in correlation coëfficients of 0.999 (plasma, range 0–1.5 mg/l), 0.999 (blood, range 0–1.5 mg/l) and 0.999 (water, range 0–9 mg/l).

3.8. Recovery

The recovery from plasma of 0.635 mg/l AZM was 103.0% ($n = 6$, vc = 1.5%) and of 1.0 mg/l CLM was 100.0% ($n = 6$, vc = 0.8%).

The recovery from blood of 0.635 mg/l AZM was 94.5% ($n = 6$, vc = 2.3%) and of 1.0 mg/l CLM was 99.4% ($n = 6$, vc = 1.6%).

Recovery of 2.0 mg/l AZM from a PMNN suspension was 103.7% ($n = 4$, vc = 3.4%).

3.9. Lower limit of quantitation

The lower limit of quantitation was 0.042 ± 0.017 mg/l (plasma, $n = 6$), 0.119 ± 0.065 mg/l (blood, $n = 6$) and 0.072 ± 0.036 mg/l (water, $n = 6$). A chromatogram of blank plasma and plasma from a patient sample with 0.038 mg/l AZM are shown in Fig. 2.

3.10. Accuracy

Six different human plasma samples were spiked with AZM to a concentration of 0.317 mg/l and were calculated on a standard curve. The concentration found was 0.317 mg/l ($n = 6$, vc = 3.2%).

3.11. Stability

The derivatized extracts were stable for at least 24 h. The stock solution of 0.2 mg/ml of AZM in methanol kept at −20 °C was stable for at least 4 months. The work solution

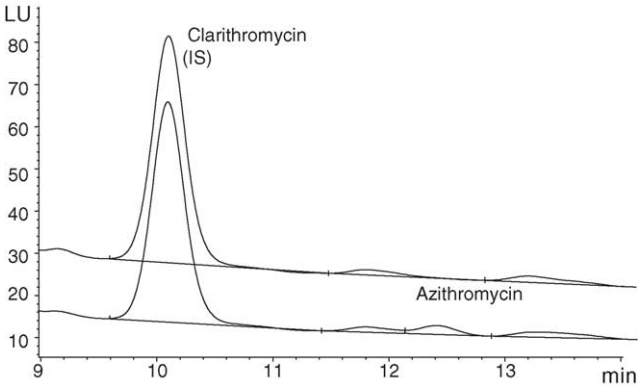

Fig. 2. Chromatogram of blank plasma (upper line) and plasma from a patient blood sample with 0.038 mg/l AZM (bottom line), which is just below the LOQ (0.04 mg/l). Clarithromycin (1 mg/l) was added to both samples as internal standard.

0.01 mg/ml of AZM in methanol kept at −20 °C was stable at least 9 months.

The plasma and blood samples were at least 18 months stable at −30 °C. Isolated PMNNs samples were at least stable for 4 months.

3.12. PMNN separation procedure

The isolation procedure was performed six-fold on a homogenised sample after addition of 23.35 μg AZM per 6 ml and a 2-h incubation period.

The homogenised blood sample contained 7.3×10^9 PMNNs per litre. With the separation procedure we harvested on average $3.7 \times 10^9 \pm 0.4 \times 10^9$ PMNNs per litre after the volume was adjusted to the starting volume (6 ml). On average, we could recover 51% (vc = 6%, $n = 6$) of the PMNNs. Flow cytometric analysis showed a 91% content of PMNNs in the cell pellet.

After determination of the volumes and the AZM concentration of all layers we could recover 88% (vc = 2.4%, $n = 6$) of the added amount of AZM. Intra-assay variation for the PMNN selection and quantification process is calculated from the PMNN fraction in which we found on average 0.42 mg AZM/l blood (vc = 7.8%, $n = 6$).

From the two patient samples, we isolated on average 52.3% of the PMNNs ($n = 8$, vc = 15%).

In patient 1, the AZM concentration in blood was 3.8 mg/l. The sum of the AZM content of all layers was on average 86.5% (vc = 2.4%, $n = 4$) of the amount detected in blood. In patient 2, we found an AZM level of 0.88 mg/l in blood and could recover 96.0% (vc = 2.3%, $n = 4$).

After correction for the incomplete PMNN harvest, we found in the PMNN fraction 1.35 mg/l blood (vc = 4%, $n = 4$) in patient 1 and 0.61 mg/l blood (vc = 14%, $n = 4$) in patient 2.

3.13. Pharmacokinetic study

The analytical method and the neutrophil separation technique were applied to a Cystic Fibrosis patient after obtaining informed consent. Fig. 3 shows the concentration–time curves of AZM in plasma (mg/l), in blood (mg/l) and in PMNNs in 1 l blood (mg AZM in PMNNs/l blood). We see a rapid decline in plasma concentration with a $t_{1/2\alpha} = 0.1$ h. The $t_{1/2\beta}$ is in plasma is 96 h, in blood 217 h and in PMNNs 340 h. At $t = 220$ h, over 88% of the blood content of AZM is internalised in PMNNs. The present detection method proved to be applicable in this pharmacokinetic study.

4. Discussion

We showed that AZM can be determined with HPLC and fluorescence detection after derivatization with FMOC-Cl in the range 0.05–1.5 mg/l. The assay was validated for use in plasma. Recovery, linearity, LOQ and inter-assay variation

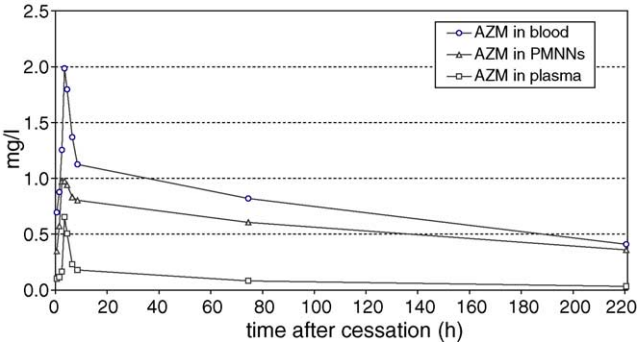

Fig. 3. Concentration of AZM in blood (circles), polymorphonuclear neutrophils (triangles) and plasma (squares) of a cystic fibrosis patient after chronic (>35 days) administration of AZM 500 mg per day and cessation of AZM therapy during a 10-day sampling period.

from blood and recovery, LOQ and intra-assay variation from isolated PMNNs were also assessed. This makes the assay useful for pharmacokinetic research in our group of patients.

FMOC-Cl is able to form a stable ester-bond with the macrolide hydroxyl group. FMOC-Cl reacts also with hydroxyl groups and with amino groups of other compounds and is used in amino-acid determination.

In order to achieve a reduction in LOQ with a smaller sample, we optimised the chromatographic conditions towards AZM using a different column and a higher water content in the acetonitril–water mobile phase compared to the method described before [10]. On molecular basis, we used an overage of FMOC-Cl compared to the molecular amount of AZM of about 500 times, calculated on an AZM concentration of 1 mg/l. Still, the added amount is critical: increase of the amount led to a small increase in AZM signal but to a higher increase in interferences, most probably due to FMOC-Cl reacting to hydroxyl- or amino-groups of other substances. Because, an increase of the added amount of FMOC-Cl led to an increase in AZM signal, we could conclude that not all AZM was derivatized or an equilibrium between underivatized and derivatized AZM had not been reached. This did, however, not influence the validation parameters, probably due to a linear level of reaction between AZM and the internal standard (CLM).

The isolation and detection procedure for isolated neutrophils is difficult to validate completely. AZM is incorporated in PMNNs. In order to validate the determination of incorporated AZM from PMNNs, we did both an ex-vivo uptake experiment and a determination in patient's samples. We quantified AZM in all separation and washing fluids. Ex-vivo we could retrieve 88% (vc = 2.4%, $n = 6$) of the AZM that was added to the blood sample. In the patient samples we could retrieve 87% (1 sample, $n = 4$, vc = 3.5%) and 96% (1 sample, $n = 4$, vc = 5.1%). This gives an indication that the separation method and subsequent analysis were reliable.

We could recover 51% of the PMNNs with a purity of 91%. In the isolation procedure, we perform a differentiated cell count before and after the separation procedure in order to quantify the recovery in every separation run and to be able to correct for incomplete recovery of PMNNs.

Ibuprofen showed some interference with the internal standard clarithromycin at a concentration of 30 mg/l. To overcome this interference the analyte could be analysed with a diode array detector instead of a fluorescence detector. When combined fluorescence and diode array detection is applied for, respectively, AZM and clarithromycin no loss in sensitivity occurred. However, in a sample obtained from a patient on ibuprofen no interference was seen due to a far lower plasma level of ibuprofen than anticipated.

We can conclude that the described HPLC method with pre column derivatization of AZM with FMOC-Cl is a valid method to determine AZM in plasma. The isolation method for PMNNs, mass density separation with Ficoll-Paque separation medium, did not interfere with the assay. The assay proved applicable for pharmacokinetic research of AZM.

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