MEPS as a Rapid Sample Preparation Method to Handle Unstable Compounds in a Complex Matrix: Determination of AZD3409 in Plasma Samples Utilizing MEPS–LC–MS–MS

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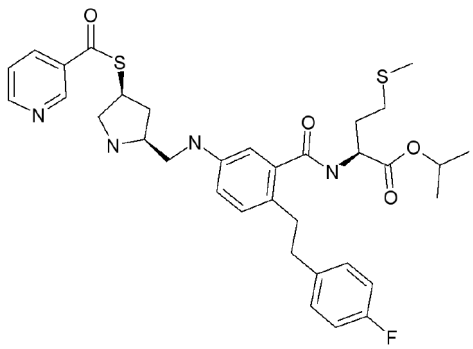
Abstract

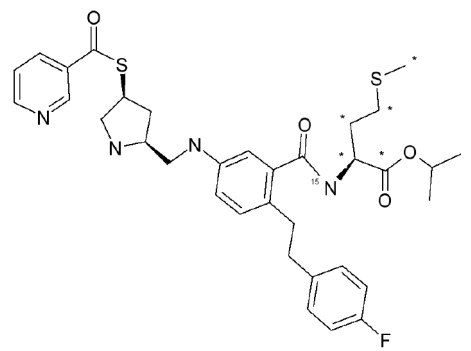
The aim of the present investigation is to develop a simple, fast, and sensitive method for the determination of a new candidate drug, AZD3409, in rat, dog, and human plasma samples. AZD3409 is stable in aqueous solutions at low pH (< 4) but not in whole blood or in plasma. In rat plasma at 25°C, more than 90% of the compound is degraded within 40 min. When 20 mg of NaF and 50 µL of protease inhibitor cocktail are added to 1.0 mL of rat blood, AZD3409 is stable for up to about 90 min. Due to the instability of AZD3409, microextraction in packed syringe (MEPS) is used as an online and fast sample-preparation method, followed by liquid chromatography–tandem mass spectrometry (LC–MS–MS) for the quantitation of this compound in plasma samples. In MEPS, the sampling sorbent is 1 mg of polystyrene polymer packed in a 250-µL syringe. When the plasma sample (50–250 µL) is withdrawn through the syringe by an autosampler, the analyte is adsorbed to the solid phase. The analyte is then eluted with an organic solvent such as methanol or the LC mobile phase (20–50 µL) directly into the instrument's injector. MEPS is rapid and easy to use. The lower limit of quantitation for AZD3409 is established to be 0.024 µM. The accuracy of the quality-control samples ranged from 89% to 102%, and the precision (C.V.%) had a value of 11–16% for the plasma samples. The calibration curve in plasma is obtained in the concentration range 0.022–9.0 µM. The coefficients of determination (R^2) for plasma samples were ≥ 0.998 for all runs. The present method is used for the analysis of rat and dog plasma samples.

Introduction

The measurement of drug levels in biological fluids is of crucial importance for drug discovery and development. In addition, it allows the optimization of pharmacotherapy and provides a basis for studies of patient compliance, pharmacokinetics, and the influence of co-medications. The sample preparation is often a limiting step to perform fast bioanalysis. In general, sampling and sample preparation steps constitute over 80% of the total analysis time, and these steps are important in determining the success of analyzing compounds of interest in complex matrices such as biological samples. Recent developments in sample-handling techniques are directed towards automation and online



AZD3409



[$^{15}N_1$, $^{13}C_5$] AZD3409 (I.S.)

Figure 1. Chemical structures of AZD3409 and internal standard.

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coupling of sample-preparation units and detection systems. In addition, miniaturization is a growing trend in the bioanalysis area, so this new method may be regarded as a step in this development.

Last decade, solid-phase microextraction (SPME) and stir-bar-sorptive extraction (SBSE) were the most attractive sample preparation methods and have been widely used for forensic, clinical, and pharmaceutical analysis. SPME as sample preparation has become a popular microextraction technique. Today, the technique is employed to extract a wide range of analytes in many areas. The extraction is based on partitioning of the analyte between the organic phase on the fused silica fiber and the analyte. Many factors, such as pH, temperature, salt concentration, and stirring, affect the equilibrium constant and the equilibration time (1–3). Microextraction in a packed syringe (MEPS) is a new miniaturized, solid-phase extraction method that can be connected online to GC or LC without any modifications (4–8). In MEPS, approx. 1 mg of the solid packing material is packed inside a syringe (100–250 µL) as a plug or between the barrel and the needle. Sample preparation takes place on the packed bed. The bed can be coated to provide selective and suitable sampling conditions. This approach for sample preparation is very promising for many reasons: (*i*) it is easy to use; (*ii*) it is a fully automated online procedure; (*iii*) it is rapid; and (*iv*) the cost of analysis is minimal compared to conventional solid-phase extraction. The MEPS technique differs from commercial solid-phase extraction (SPE) in that the packing is inserted directly into the syringe, not into a separate column. Thus, there is no need for a separate robot to apply the sample into the solid phase as with conventional SPE. The packed syringe can also be used several times, more than 100 times with plasma or urine samples, and more than 400 times for water samples, whereas a conventional SPE column can only be used once. Compared with SPME, the new technique is more robust. In SPME, the sampling fiber of SPME is quite sensitive to the nature of the sample matrix. The new technique can be used for complex matrices without problems (such as plasma, urine, and organic solvents), which is not the case with SPME. Also, much higher extraction recovery can be obtained (60–90%) compared to SPME (1–10%). Small sample volumes can be treated (10 µL) compared to SPME (≥ 1000 µL).

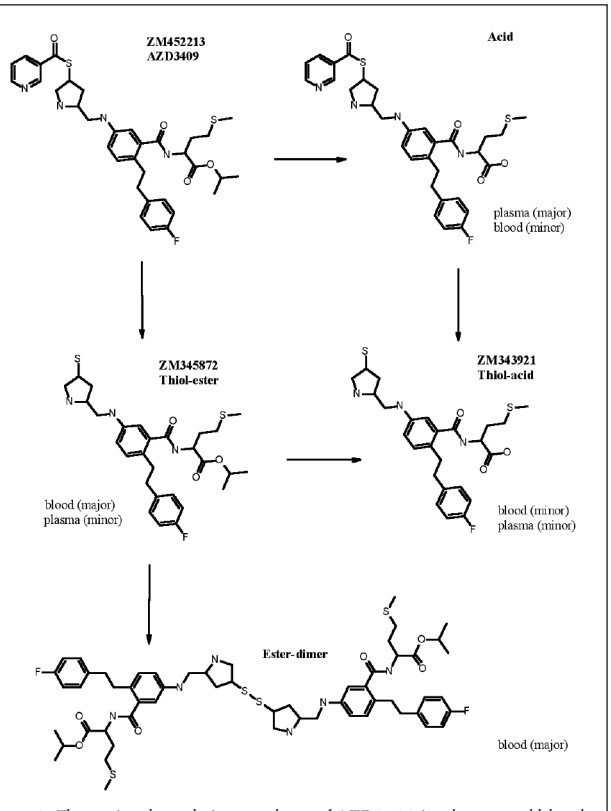

Figure 2. The major degradation products of AZD3409 in plasma and blood.

There is a great demand for novel chemotherapeutic agents in the treatment of bone metastases in prostate cancer. AZD3409 (Figure 1) is a novel oral protein prenyl transferase inhibiting both farnesyl transferase and the geranylgeranyl transferase-1. It was found that AZD3409 is active in preclinical in vitro and in vivo models (9). AZD3409 is a double pro-drug, its metabolism involves conversion to a thiol ester intermediate (ZM345872), then, intracellularly to a thiol acid (ZM343921) active moiety (Figures 1 and 2).

Bioanalysis of the parent compound (AZD3409) is problematic due to its instability in rat blood and plasma. In this validation, the stability of AZD3409 in rat blood and plasma has been studied. The bioanalytical method using LC–MS–MS has been set up, and the methodology has been validated. The accuracy and precision were determined at three different concentration levels (QC samples).

Experimental

Chemicals

Dimethyl sulphoxide (DMSO), sodium fluoride (NaF), and protease inhibitor cocktail (Sigma P8340) were purchased from Sigma-Aldrich (Stockholm, Sweden). AZD3409 was prepared as stock solution in DMSO (10 mg/mL) and stored at –20°C.

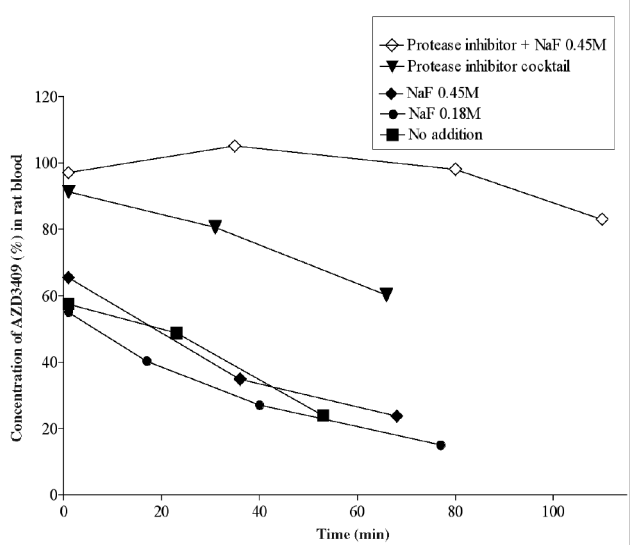

Figure 3. Stability of AZD3409 in rat blood with and without addition of NaF and protease inhibitor at 0°C.

AZD3409 and [$^{15}N_1$, $^{13}C_5$] AZD 3409 (I.S.) (Figure 1) were supplied by the Department of Medicinal Chemistry, AstraZeneca (Alderley Park, UK). Acetonitrile, methanol, formic acid and ammonium hydroxide were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

Apparatus

The LC instrument included two pumps, Shimadzu LC-10Advp (Kyoto, Japan), an autosampler, CTC-Pal from Crelab (Knivsta, Sweden) and a 20-µL sample loop. A Zorbax SB-C8, 3.5 µm (50 × 2.1 mm) column obtained from Agilent (Palo Alto, CA) was used as an analytical column connected to an Optiguard (C_8, 10 × 1 mm) as a guard column. A Valco C4W valve from Valco Instruments (Houston, TX) was used as gate valve between the liquid chromatograph and the mass spectrometer. The Milli-Q water was obtained using a Reagent Grade Milli-Q Plus water purification system from Millipore Corporation (Bedford, MA). A centrifuge, Hettich Rotanta/AP (Tuttlingen, Germany), was used for plasma centrifugation.

A gradient HPLC pump was used with mixer volume of 0.1 mL. Mobile phase A was acetonitrile–0.1% formic acid in water (10:90, v/v), while mobile phase B contained acetonitrile–0.1% formic acid in water (80:20, v/v). The gradient started from 0% of phase B up to 80% from 1 to 5 min and then from 5 to 6 min isocratic at 80% of phase B, and at 6.1 min phase B was set to 0% again. For system stability, the next injection was performed after 8 min. The flow rate was 150 µL/min, and sample volume (loading) was 50 µL.

All experiments were conducted using a triple quadrupole mass spectrometric instrument Micromass QII Z-spray (Waters, Manchester, UK) equipped with a Z-electrospray interface operated in positive ion mode. The parameter settings used were: capillary voltage at 3.1 kV, cone voltage at 38 V, extractor at 5 V, RF lens at 0.2 V, source block, and desolvation temperatures at 150°C and 300°C, respectively. Nitrogen was used both as drying (400 L/h) and nebulizing gases (20 L/h), the vacuum was 2×10^{-5} mbar in the mass analyzer and 2×10^{-3} mbar in the collision cell. Argon was used as collision gas and collision energy was 25 eV. The gases were from ScanGas (Stockholm, Sweden). The data were collected and processed using MassLynx version 3.5 and all calculations were based on peak area ratios.

The scan mode was multiple reaction monitoring (MRM) using precursor ion at $[M+H]^+$ (*m/z:* 654 and 659.5), and after collisional dissociation the product ions 462 and 462 were used for quantification of AZD3409 and [^{15}N,$^{13}C_5$]AZD3409 (IS), respectively.

Preparation of stock and standard solutions

Plasma samples were stored at –20°C. Before use, the plasma was thawed at room temperature and centrifuged at 3500 rpm for 10 min. Two stock solutions of AZD3409 in DMSO were prepared (one for preparation of standards and one for preparation of QC samples). From the stock solution of AZD3409 a stepwise dilution series was performed in water. Spiked plasma samples were prepared by adding 5–50 µL of analyte standard to 0.5–1.0 mL of plasma and 25 µL of the internal standard (approx. 80 µM).

MEPS–conditions

MEPS was performed using a 250-µL gas-tight syringe. Different sorbents (C2, C8, and polystyrene polymer) were tested. The polystyrene polymer, ISOLUTE ENV+ (cross-linked hydroxylated polystyrene-divinylbenzene copolymer sorbent), from Biotage (Uppsala, Sweden) gave the best recovery. This sorbent has irregular particles of average size 50 µm and nominal 60 Å porosity. One milligram of the solid material was manually inserted into the syringe as a plug. The sorbent material was tightened by filters in order to avoid movement inside the syringe.

Before being used for the first time, the sorbent was manually conditioned with 50 µL of methanol, followed by 50 µL of

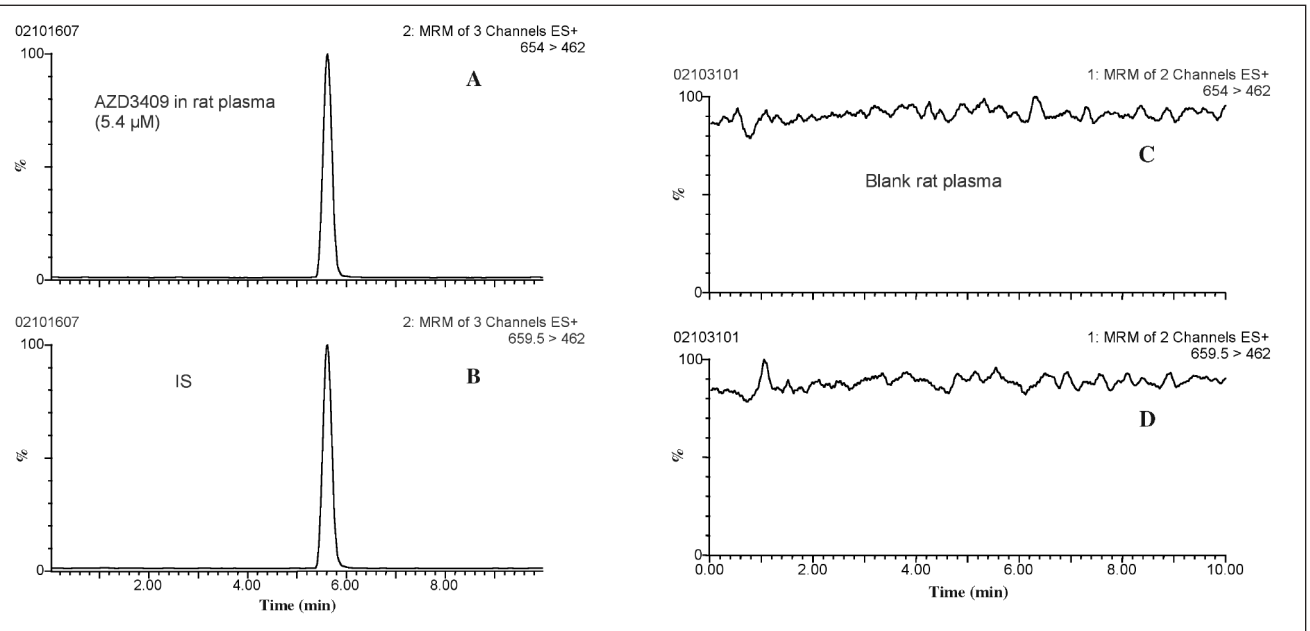

Figure 4. Mass chromatogram obtained from spiked rat plasma of a quality-control sample (5.4 µM AZD3409) (A and B) and blank plasma sample (C and D).

water–methanol (90:10, v/v). The syringe was then connected to the autosampler and the spiked plasma sample (50 µL) was withdrawn into the syringe by the autosampler. It is important that the plasma samples are withdrawn slowly (20 µL/s) and carefully to obtain good percolation between sample and solid support. The sorbent was then washed once with 100 µL of water–methanol (90:10, v/v) to remove proteins and other interferences. The analytes were then desorbed by 25 µL of methanol–water (95:5, v/v) directly into a gate valve situated between the liquid chromatograph and the tandem mass spectrometer. Cleaning of the sorbent was carried out using a 5 × 50-µL elution solution followed by 5 × 50 µL of the washing solution between every extraction. This step decreased memory effects, but also functioned as conditioning step before the next extraction. The same packing bed was used for about 100 extractions before being discarded.

To optimize microextraction in a packed syringe, factors affecting recovery such as the composition of the washing solution and elution solutions were studied.

MEPS washing

After being introduced into the syringe, the sample (50 µL) was washed once with 100 µL of the washing solution. The effect of different washing solutions on recovery was investigated. The recovery was measured as the response of a processed, spiked plasma sample expressed as peak area and calculated as the mean of three different experiments. The use of methanol in the washing mixture slightly affected the loss and the recovery of the analyte. Increasing methanol from 0% to 10% in the washing solution (water) did not increase the loss of the analyte. However, increasing it to 20% significantly increased the loss by about 10%. The best results in terms of clean extracts and recovery were obtained using 100 µL of water–methanol (90:10, v/v).

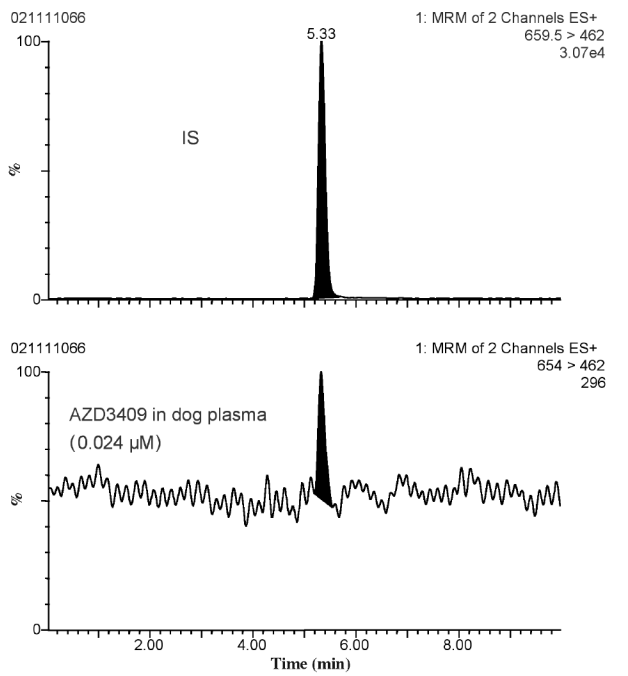

Figure 5. Representative calibration sample in dog plasma at approximately LLOQ, 0.024 µM AZD3409 with internal standard.

MEPS elution solvent

To study the recovery, solutions containing methanol, water, formic acid and ammonium hydroxide were investigated. After introduction of the sample (50 µL) into the syringe and washing with 100 µL of water–methanol (90:10, v/v), the elution efficiency was measured and compared to that of pure standard solution (100 ng/mL). The eluting efficiency increased significantly with increasing methanol content in the eluent, while the use of formic acid or ammonium hydroxide did not affect the recovery of the drug. Acceptable recovery (50 ± 3%) and pure samples were obtained using a solution of methanol–water (95:5, v/v).

Results and Discussion

Stability of AZD3409

Stability in rat blood

AZD3409 is unstable in rat blood and 30–40% was lost after only one minute when neither NaF salt nor protease inhibitor cocktail (Sigma P8340) was added at 0°C (Figure 3). The proposed structures of the major degradation products of AZD3409 in plasma and blood are given in Figure 2. In plasma, the major product was the loss of the isopropyl group from the carboxylate ester and only small amounts of the "thiol-ester" ZM345872 and "thiol-acid" ZM343921 were observed. In blood the major pathway was loss of pyridine from the thio-ester, yielding ZM345872, which was further converted to the corresponding dimer. Also, in blood, small amounts of "thiol-acid" ZM343921 and "acid" (loss of isopropyl) were observed. However, approx. 40% remained after 90 min, when 20 mg of NaF was added to 1.0 mL of rat blood. When 20 mg of NaF and 50 µL of protease inhibitor cocktail were added to 1.0 mL of rat blood, the AZD3409 was stable for up to about 90 min (Figure 3). These conditions were applied to the analysis of plasma samples. Due to the instability of AZD3409, only a few samples could be prepared at the same time (10 samples were prepared and injected before preparation of the next samples could be started).

Table I. Back-Calculated Concentration of the Calibration Samples as a Percentage of Nominal Value

Nominal Concentration (µM)	Accuracy (assay 1) (%)	Accuracy (assay 2) (%)	Accuracy (assay 3) (%)	Accuracy (assay 4) (%)
0.022	–*	99	95	92
0.045	–*	92	99	95
0.090	113	94	93	80
0.180	83	114	–*	111
0.449	116	105	108	106
0.899	89	100	106	118
1.799	97	96	103	97
4.498	104	101	95	99
8.995	99	100	101	103

* Missing due to a technical problem.

Stability in rat and human plasma

A preliminary study showed that more than 90% of the compound in rat plasma is degraded within 40 min at 25°C. When NaF was added (0.18 M in the plasma sample) and the sample temperature was 1°C, the loss of the compound decreased (74% remained after 45 min).

The stability of AZD3409 in rat and human plasma at –70°C was also investigated after addition of 20 mg of NaF and 50 µL of protease inhibitor cocktail (Sigma P8340 / diluted 1:10 in water when plasma is used) to 1.0 mL of plasma sample. The AZD3409 compound in plasma was stable at –70°C for at least 7 days.

Stability in phosphate buffer and DMSO

AZD3409 was most stable at pH 3.5, with an extrapolated $t_{1/2}$ of around 85 days at 25°C, while it has a $t_{1/2}$ of 36 h at pH 7.34 and 25°C. AZD3409 was also stable in DMSO at 4°C for at least 20 days.

Method validation

The ratios of peak areas of AZD3409 and the internal standard were measured and a standard curve without the zero concentration was constructed. Due to the complexity of a sample matrix such as plasma and the low capacity of the microextraction methods, a quadratic calibration curve is recommended for quantitation. It minimizes the percent relative error in the back-calculated values of the calibration points. Also, our test in this study showed that quadratic calibration curve gave acceptable accuracy comparing to linear calibration curve.

Calibration curves were typically described by the equation:

$$y = Ax^2 + Bx + C$$

where y is the peak-height ratio, x is the concentration of analyte, B and C are the slope and intercept, respectively, and A is the curvature. The calibration curves were weighted (1/×). Calibration standard solutions (9 concentrations) in human plasma were prepared in a concentration range of approx. 0.022–9.0µM.

Accuracy was defined as the degree of deviation of the determined value and the nominal value: [(measured value) / (nominal value)] × 100. Precision (C.V.%) was defined as the percentage of standard deviation of the observed values divided by their mean values: [(Standard deviation) / mean value] × 100. Selectivity, linearity, accuracy, precision, recovery and limit of quantitation were studied according to Shah et al. (10).

Table II. The Between-Batch Accuracy and Precision of Quality-Control Samples

Plasma samples	Mean accuracy (%) (n = 12)	Precision* (%) (n = 12)
QCL (0.11 µM)	89	14
QCM (0.45 µM)	91	11
QCH (5.42 µM)	102	16

* Precision (C.V.%) was defined as the percentage of the standard deviation of the observed values divided by their means.

Table III. The Within-Batch Accuracy and Precision of Quality-Control Samples

Batch Rat plasma	QCL (µM) (n = 6)	QCM (µM) (n = 6)	QCH (µM) (n = 6)
Mean Accuracy (%)	87	86	115
Precision* (%)	15	7.0	12

* Precision (C.V.%) was defined as the percentage of the standard deviation of the observed values divided by their means.

Calibrations

Calibration curves of plasma samples spiked with AZD3409 standards were obtained in the range 0.022–9.0 µM, with [$^{15}N_1$, $^{13}C_5$] AZD3409 as internal standard. The coefficients of determination (R^2) were 0.9991, 0.9999, 0.9991, 0.9980, and 0.9999 using plasma samples. Table I shows the back-calculated values of standard calibration samples in rat plasma (assays 1 and 2) and human plasma samples (assays 3 and 4).

Selectivity

The method selectivity was defined as non-interference with the endogenous substances in the regions of interest. LC–MS–MS analysis of the blank plasma samples showed no signal > 20% of the LLOQ. Representative chromatograms of blank plasma and spiked plasma are presented in Figures 4A and 4B.

Accuracy and precision

The QC samples were prepared in rat plasma (assays: A and B), dog plasma (assay: C), and human plasma (assay: D). The accuracy is determined as the percentage difference from the nominal concentration value for triplicates of QC samples at three different concentration levels (Table I). The between-batch mean accuracy ranged from 89% to 102%. The precision is determined by the percentage of the relative standard deviation (RSD) of the between-batch variations at three different concentration levels (QC samples). The data of between-batch variation of the precision were in the range 11–16%. The between-batch accuracy and precision results are summarized in Table I. The within-batch mean accuracy ranged from 86% to 115%. The data of within-batch variation of the precision (C.V.) were in the range 7.0–15%. The within-batch accuracy and precision results are summarized in Table III.

Lower limit of quantitation and carry-over

The carry-over was investigated by injecting elution solution after the highest standard concentration, which was lower than 0.1%. However, no carry-over was observed after several washings (five times). The lower limit of quantitation (LLOQ) in plasma was measured at 0.024µM. The precision (C.V.) was 10% (n = 6). A chromatogram of calibration sample at the LLOQ in dog plasma is shown in Figure 5.

Application of the method

The method was applied for the analysis of plasma samples from pre-clinical studies. The analysis of the rat and dog plasma

samples showed that the concentration of AZD3409 in the rat plasma samples were below the LOQ. The in vivo conversion of AZD3409 was expected to be very rapid, but due to regulatory demands it was necessary to provide a validated method for the parent compound.

Concluding remarks

An LC–MS–MS method for the assay of AZD3409 in plasma samples has been developed and validated. The results show that the method is selective and accurate. Microextraction in packed syringe is a new sample-preparation method suitable for unstable compounds in complex matrices since it is fully automated and fast. It has thus been shown that only small sample volumes are required. It is of great importance to use a suitable analytical method for both pre-clinical and clinical studies for newly introduced drugs.

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