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Simultaneous determination of AZD1152 (prodrug) and AZD1152-hydroxyquinazoline pyrazol anilide by reversed phase liquid chromatography

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ABSTRACT

A simple, selective and sensitive reversed phase liquid chromatography method utilizing ultraviolet detection has been developed and validated for the simultaneous determination of the prodrug AZD1152 and its active product AZD1152-hydroxyquinazoline pyrazol anilide (hQPA) in human and mouse plasma and mouse tissues. Isocratic separation was achieved using an 5 μm UptiSphere HDO C-18 column (150 mm × 4.6 mm) with guard column in combination with a mobile phase comprised of phosphate buffered water (50 mM; pH 3.4) and acetonitrile (81.5: 18.5; v/v). UV detection at 318 nm was used. Sample preparation involved a single-step protein precipitation with ethanol. *Ex vivo* conversion of AZD1152 by endogenous phosphatases was prevented by immediate cooling of the samples in ice-water and addition of sodium fluoride and EDTA. The validation parameters included specificity, recovery, accuracy, precision, sensitivity and stability. The lower limit of quantification in human plasma for AZD1152 and hQPA was 25 ng/ml. The applicability of the method was demonstrated by successful determination of AZD1152 and hQPA in human plasma and in plasma, brain, liver, kidney and ileum samples from mice dosed with AZD1152.

1. Introduction

Aurora B kinase is a member of the Aurora kinase family which is an important part of the chromosomal passenger complex (CPC) and as such a key mitotic regulator required for histone H3 phosphorylation, chromosome bi-orientation, the spindle assembly checkpoint and cytokinesis [1–4]. Furthermore, Aurora B is frequently amplified in many human cancer cells and its inhibition induces a profound mitotic phenotype [2,5]. Aurora B deficient cells exit mitosis without actually dividing into two daughter cells and return to G1 with a tetraploid DNA content leading to rapid loss of proliferative potential [6].

AZD1152 (Fig. 1) is a dihydrogen phosphate prodrug and has a high aqueous solubility of 10 mg/ml in simple pH-adjusted saline. In plasma, it is rapidly converted into the active species AZD1152-hydroxyquinazoline pyrazol anilide (hQPA). This compound is the first of a novel series of inhibitors that shows up to 1000-fold higher selectivity for Aurora kinase B over Aurora kinase A [7]. In several human tumor xenograft mouse models AZD1152 induced time dependent pharmacodynamic changes consistent with inhibition of Aurora B kinase [8]. This striking *in vivo* activity against multiple tumor types has led to the current investigation of this novel agent in phase I clinical trials [7].

ATP-binding cassette (ABC) transporters are a family of drug transporting proteins that are involved in both the uptake and elimination of drugs as well as in causing multidrug resistance [9,10]. Recently the aurora kinase inhibitor JNJ-7706621, which has advanced into clinical trials, was shown to be a substrate for breast cancer resistance protein (BCRP/ABCG2) [11] a member of the ATP-binding cassette (ABC) transporter family [12–15]. Currently we are investigating the importance of ABCB1 (P-glycoprotein) and ABCG2 in the disposition of AZD1152 and hQPA by *in vitro* and *in vivo* experiments. For this purpose, we have available a set of mouse models which are deficient in Abcb1 and/or Abcg2 [11–16]. However, a method for the bioanalysis of AZD1152 and hQPA in mouse tissues has not been published. We present in this paper the development and validation of an HPLC–UV method for the simultaneous determination of both AZD1152 and hQPA in human plasma and in murine plasma and tissues (brain, kidney, liver, lung and small intestine). These tissues were selected because it is known that they

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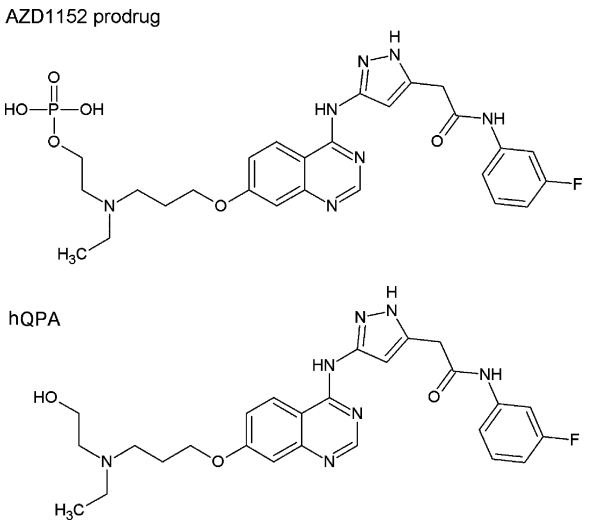

Fig. 1. Molecular structures of AZD1152 and hQPA.

express ABC-transporters and fulfill a protective role in defending the body against the toxic action of anticancer drugs [17]. The applicability of the method was demonstrated in a pilot *in vivo* pharmacokinetic study in mice.

2. Materials and methods

2.1. Reagents and chemicals

AZD1152 dihydrogen phosphate pyrazoloquinazoline prodrug trihydrate (AZD1152) and AZD1152-hydroxyquinazoline pyrazol anilide (hQPA) (Fig. 1) were a generous gift from Astrazeneca Pharmaceuticals (Macclesfield, UK). Acetonitrile of HPLC grade, potassium-dihydrogenphosphate (KH_2PO_4), and di-potassium hydrogen phosphate of pro analysis grade, were purchased from Merck (Darmstadt, Germany). Phosphate buffered saline (PBS), pH 7.4, contained sodium chloride (8 g/l), potassium chloride (0.2 g/l), disodium hydrogen phosphate (1.15 g/l), and KH_2PO_4 (0.2 g/l). The water used was of Milli-Q grade purified by a Milli-Q plus purification system (Millipore, Bedford, USA). Human plasma was obtained from Sanquin (Amsterdam, the Netherlands). Ethanol and bovine serum albumin (BSA) originated from Roche (Woerden, the Netherlands). Methotrexate (MTX) used as internal standard was obtained as Emthexate® from the pharmacy of the Slotervaart hospital, Amsterdam, the Netherlands.

2.2. Treatment of animals and sample processing

The institutional Animal Experiments Committee approved the study protocol. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Animals used in this study were female FVB mice between 9 and 14 weeks of age. Animals were kept in a temperature controlled environment with a 12-h light/12-h dark cycle, and received a standard diet (AM-II; Hope Farms, Woerden, The Netherlands) and acidified water *ad libitum*.

AZD1152 was administered as an intra-peritoneal *bolus* dose to a total of nine mice at a dose of 100 mg/kg. This route of parental drug administration allows serial blood sampling from the tail vein without the possible contamination from persistent interstitial drug that may be present at the injection site after intravenous administration via the tail. Three mice were sampled at 0.5, 1 and 2 h. Three other mice were sampled at 2, 4 and 6 h. Another three mice were sampled at 6, 8, 24 h. At these time points 75 μl whole blood samples were drawn from the tail vein in heparinized capillaries. The blood sample was immediately transferred to 0.5 ml eppendorf tubes containing 50 μg NaF and 87.5 μg Na_2EDTA, vortexed for 10 s at low speed, and centrifuged at 20,000 × *g* at 0 °C for 5 min. In order to have the same total amount of plasma per sample as used for the calibration curve, 35 μl of mouse plasma was supplemented with 65 μl of human plasma, and immediately processed with ethanol, as will be described later. This deproteinization step prevents further dephosphorylation of AZD1152 by phosphatases present in plasma. To show the applicability of the assay for tissues the mice were anesthetized with methoxyflurane and sacrificed by cervical dislocation after the last blood sampling at time points 2, 6 and 24 h. Brain, liver, both kidneys, and ileum were removed and placed in a tube on ice. The collected tissue were weighed immediately, followed by homogenization with a Polytron PT1200 (Kinematica AG, Littau, Switzerland) on ice in 3, 5, 3, and 2 ml of ice-cold 4% BSA solution containing 25 mM NaF and 5 mM EDTA, respectively, directly followed by processing of 100 μl tissue homogenate with ethanol for HPLC measurement, as will be described for human plasma below. After processing plasma and tissue samples were stored for 1 day at −20 °C until measurement by HPLC–UV.

2.3. Inhibition of phosphatase activity in mouse plasma and liver homogenate

To inhibit phosphatase activity human and mouse plasma and all mouse tissue homogenates were prepared so that they contained 25 mM NaF and 5 mM ethylene diamine tetra-acetic acid (EDTA). These inhibitors and concentrations were proposed by AstraZeneca on basis of their research. Inhibition of AZD1152 dephosphorylation was tested in triplicate on ice and at ambient temperature with plasma and liver homogenates from three different mice by incubation of 100 μl mouse plasma (diluted 10-fold with PBS) or 100 μl liver homogenate spiked with 1 μg internal standard, and AZD1152 at 50 μg/ml. Subsequently, reactions were stopped at 0, 0.25, 0.5, 1, 4, 8, and 24 h by adding 400 μl 100% ethanol, after which samples were processed for HPLC measurement.

2.4. Extraction of samples with ethanol

A volume of 100 μl of plasma or tissue homogenate spiked with 50 μl of internal standard solution (20 μg/ml MTX) was mixed with 400 μl of ethanol. After mixing for 10 s the samples were stored at −20 °C for 2 h [18]. Next, the samples were centrifuged at 12,000 × *g* for 10 min (4 °C) and 500 μl supernatant was transferred to a clean 1.5 ml vial and dried for 4 h by vacuum concentration (SpeedVac; Savant, Rarmindale, NY, USA) at ambient temperature. The pellet was reconstituted in 150 μl PBS (containing 15% acetonitrile) by sonication for 15 min. The solution was centrifuged at 12,000 × *g* for 10 min and the supernatant (∼140 μl) was transferred to an HPLC vial for injection.

2.5. HPLC system

The liquid chromatography system consisted of an Ultimate 3000 Pump (Dionex, Sunnyvale, USA), an ASI-100 Automated Sample Injector (Dionex, Sunnyvale, USA) provided with a 250 μl sample loop, and a 759A Absorbance Detector (Applied Biosystems, Foster City, USA) with detection wavelength set at 318 nm. For some experiments we have used a model 996 Photodiode Array Detector (Waters, Milford, USA). Chromatographic separations were carried out at ambient temperature using an Uptisphere HDO C18 column, 5 μm, 150 mm × 4.6 mm preceded by an Uptisphere HDO C18 guard column, 5 μm, 20 mm × 4.6 mm (Interchim, Montlucon, France). The mobile phase consisted of 50 mM potassium

dihydrogen phosphate buffer adjusted to pH 3.5 by 37% HCl and acetonitrile (81.5: 18.5%; v/v). The flow rate was set at 1.0 ml/min. Chromatographic data acquisition and reprocessing was performed using Chromeleon version 6.80 (Dionex Corp. Sunnyvale, CA, USA).

2.6. System suitability

The column was initially equilibrated with mobile phase to get a stable baseline. A blank sample followed by two system suitability samples containing a mixture of AZD1152 and hQPA at 1000 ng/ml in mobile phase were injected and chromatograms recorded.

2.7. Method validation

Method validation was using the rules set forward by the European Pharmacopoeia [19] and the International Conference on Harmonization (ICH) guidelines Q2A and Q2B [20,21] as guidance.

2.8. Calibration, internal standards and quality controls

We prepared two independent primary AZD1152 standard stock solutions of 10 mg/ml in a mixture containing 0.9% NaCl and 35 mM $Na_3PO_4.12H_2O$ (pH 9). Also, two independently weighed primary hQPA standard stock solutions of 10 mg/ml were prepared in DMSO. These AZD1152 and hQPA primary stock solutions were diluted to 100 μg/ml secondary standard stock solutions in PBS containing 15% acetonitrile, or in 100% acetonitrile, respectively. Primary and secondary stock solutions were stored at −20 °C and thawed at 37 °C for daily fresh preparation on ice of 10,000, 1000 and 100 ng/ml working solutions containing both AZD1152 and hQPA in ice-cold phosphatase inhibited human plasma (containing 25 mM NaF and 5 mM EDTA). From these solutions a calibration curve was prepared on ice in duplicate just before each run at 25, 50, 100, 250, 500, 1000, 5000, 10,000 ng/ml by dilution with phosphatase inhibited human plasma. Quality controls at 100, 1000 and 10,000 ng/ml in phosphatase inhibited human plasma were prepared in duplicate from AZD1152 and hQPA standard stock solutions independently weighed from the calibration standards. MTX internal standard stock solution of 20 μg/ml was prepared in Milli-Q water and stored at −20 °C. A volume of 50 μl, corresponding to 1 μg of MTX, was added as internal standard to 100 μl of standard sample and, next, samples are extracted as described previously.

2.9. Specificity

Blank human and mouse plasma and mouse tissues from three different humans and mice were processed in triplicate to identify possible interferences from endogenous components present in the biological matrices.

2.10. Recovery

Recovery of AZD1152, hQPA and MTX was determined by comparing the mean concentrations measured in processed plasma and tissue samples spiked with stock standard solutions relative to unprocessed aqueous samples spiked at the same concentration.

2.11. Within- and between-day precision, accuracy, and recovery

Human and mouse plasma and mouse tissues homogenates were spiked in triplicate with either AZD1152 or hQPA at 100, 1000, and 10,000 ng/ml and immediately processed on ice. Human plasma samples were measured in triplicate on three consecutive days. The between-day (BDP) and within-day precision (WDP) was calculated by one-way analysis of variance (ANOVA) for each control sample using the run day as classification variable using the software package SPSS for windows (version 15.0; SPSS, Chicago, IL, USA). The day mean square (DayMS), error mean square (ErrMS) and the grand mean (GM) of the observed concentrations across run days were used. The WDP% and BDP% for each QC sample was calculated using the formulas:

$$\mathrm{WDP\%} = \frac{(\mathrm{ErrMS})^{0.5}}{\mathrm{GM}} \times 100\%$$

$$\mathrm{BDP\%} = \frac{[(\mathrm{DayMS} - \mathrm{ErrMS})/n]0.5}{\mathrm{GM}} \times 100\%$$

where n is the number of replicates within each run.

$$\mathrm{The\ accuracy} = \frac{\mathrm{GM}}{\mathrm{nominal\ concentration}} \times 100\%$$

Mouse plasma and tissues were measured in triplicate in one run day only.

$$\mathrm{The\ accuracy} = \frac{\mathrm{mean\ concentration}}{\mathrm{nominal\ concentration}} \times 100\%$$

$$\mathrm{The\ WDP} = \frac{\mathrm{SD}}{\mathrm{mean\ concentration}}$$

2.12. Lower limit of detection (LLOD) and lower limit of quantification (LLOQ)

Blank human and mouse plasma and mouse tissues were spiked with internal standard and processed in triplicate to assess the limit of detection, defined as the amount that could be detected with a signal to noise ratio of 3.

The LLOQ is determined by spiking human plasma from six different individuals in threefold with 25 or 30 ng/ml AZD1152 or hQPA. The LLOQ is defined as the concentration that can be determined with a precision of better than 20% and an accuracy between 80 and 120% of the nominal value.

2.13. Sample stability

Three different batches of ice-cold human plasma were spiked with AZD1152 and hQPA at 100 or 10,000 ng/ml. Spiked plasma samples were stored unprocessed, or after immediate processing with ethanol as described earlier, in triplicate for 0, 12, 24 and 48 h at −20 °C. We also assessed the stability of AZD1152 and hQPA in processed samples placed in the autosampler, and measured immediately and after 12, 24, and 48 h. Another batch of processed samples was placed at −20 °C for 0, 1, 2 and 3 months. These samples were thawn at room temperature and analyzed immediately by HPLC–UV.

3. Results

3.1. Method development

Chromatographic behaviour of AZD1152 and hQPA on a classic reversed phase packing material (e.g. Waters Symmetry® C18, 5 μm, 150 mm × 4.6 mm) was not satisfactory due to extensive peak tailing and low column efficiency (plate numbers for AZD1152 and hQPA of 564 and 1484, respectively). Switching to an Uptisphere C18 reversed phase HDO HPLC column (5 μm, 150 mm × 4.6 mm) yielded greatly improved efficiencies for both AZD1152 and hQPA. The peak symmetry of AZD1152 was still subject to some tailing, which could be completely suppressed by using 50 mM phosphate buffered mobile phase resulting in plate numbers of 8000 for both AZD1152 and hQPA.

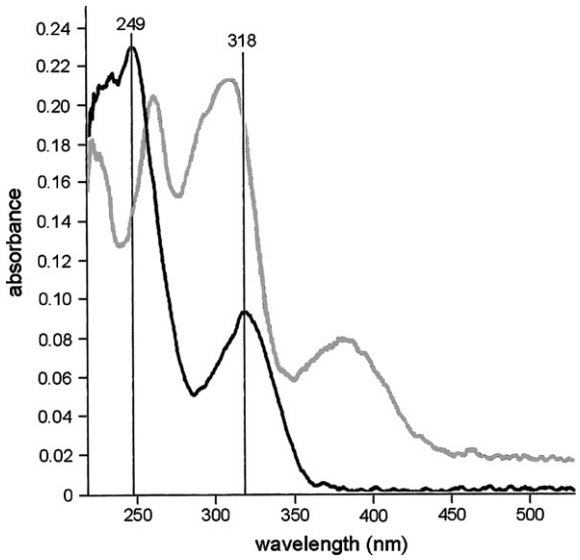

Fig. 2. Absorbance spectrum (200–550 nm) of AZD1152 and MTX, black and grey lines, respectively. The absorbance maxima of AZD1152 at 249 and 318 nm are indicated.

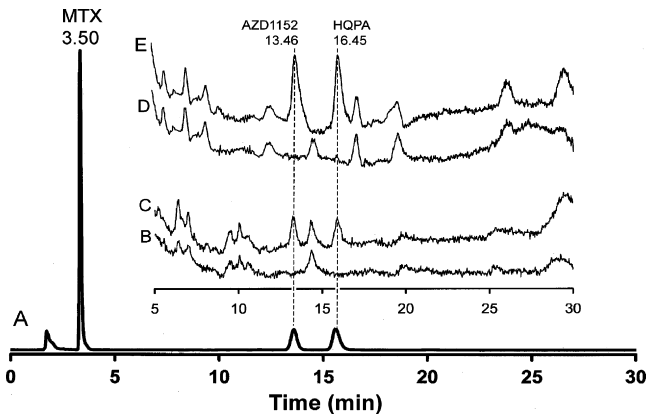

Fig. 3. Representative chromatogram (A) of blank human plasma spiked with 10,000 ng/ml AZD1152 and hQPA. The insert contains stacked chromatograms of (B) blank human plasma; (C) blank human plasma spiked with AZD1152 and hQPA at 25 ng/ml; (D) blank mouse plasma and (E) blank mouse plasma spiked with AZD1152 and HQPA at 100 ng/ml. AZD1152, hQPA and MTX are annotated with their retention times.

The pH value of the HPLC mobile phase was modified to achieve a good separation between AZD1152 and hQPA. AZD1152 and hQPA both have a quinazoline (p*K*a 5.11) and a N-ethylethanolamine (p*K*a 9.88) group that are protonated at pH 3.4. In addition AZD1152 contains a phosphate group with p*K*a values of 5.92 and 2.0 resulting in a negative charge at pH 3.4. In order to allow isocratic separation, the difference in polarity between AZD1152 and hQPA should not be too large. Good separation of AZD1152 and hQPA was achieved using a mobile phase with a pH 3.4.

Finally, the fraction of acetonitrile in the mobile phase was set to 18.5%, this achieved a good separation of AZD1152 and hQPA from endogenous substances with an acceptable overall analysis time of 30 min. No interfering late-eluting peaks were observed.

3.2. Specificity

AZD1152 and hQPA have similar UV spectra with absorbance maxima at 249 and 318 nm. Although the molar extinction coefficient is about 50% lower at 318 nm compared to 249 nm (Fig. 2), we have used detection at 318 nm since this increases the selectivity towards matrix impurities remaining after sample processing. We checked the usefulness of MTX as internal standard. MTX has good absorbance at 318 nm (Fig. 2), and with a retention time of 3.50 min, it was well separated from the bulk of endogenous interferences which eluted mostly earlier, and AZD1152 and hQPA, which eluted at 13.46 and 16.45 min, respectively. Fig. 3 shows representative chromatograms of blank human and mouse plasma without and after spiking with 25 or 100 ng/ml of both AZD1152 and hQPA, respectively.

No interfering peaks from endogenous substances were present at the retention times of both compounds.

3.3. Range and linearity

An eight-point calibration curve was constructed for both compounds at the specified concentrations. This concentration range was selected based on drug concentrations observed in *in vivo* samples measured in a pilot studies. The calibration curve was calculated using weighted (x^{-1}) least square linear regression analysis and yielded high correlation coefficients (Table 1). These calibration curves in human plasma were used to quantify the concentrations of AZD1152 and hQPA concentrations in the different matrices. Similar data for linearity, slope and correlation coefficient of the calibration curves were obtained when using external calibration (i.e. without the use of MTX as internal standard, which was not unexpected given the relatively simplicity of the sample pretreatment procedure. Nevertheless, we preferred using internal calibration with MTX as internal standard.

3.4. Limit of detection and lower limit of quantification

The limit of detection (LLOD) was about 15 ng/ml in all matrices. The lower limit of quantification (LLOQ) was determined in six different human plasma samples spiked ($n = 3$) with a nominal concentration of AZD1152 or hQPA. The LLOQ is defined as the concentration that can be determined with a precision of better than 20% and an accuracy between 80 and 120% of the nominal value. The determined LLOQ for both AZD1152 and hQPA was set at 25 ng/ml. At this LLOQ the accuracy was 92.6% and 116.2% and the precision 10.2% and 18.9% for AZD1152 and hQPA, respectively.

3.5. Within- and between-run precision, accuracy and recovery

The accuracy and precision for AZD1152 and hQPA in human plasma was determined by analyses of samples spiked at three concentrations. These analyses were performed in triplicate in four

Table 1
Regression analysis[a] of calibration curves for AZD1152 and hQPA in the specified concentration range.

Compound	Range (ng/ml)	Intercept[b] ($a \pm$ S.D.)	Slope[b] ($b \pm$ S.D.)	Correlation coefficient[c] (r)
AZD1152	25–10,000	0.01813 ± 0.00686	0.004567 ± 0.0001528	0.99963
hQPA	25–10,000	0.04477 ± 0.01504	0.005967 ± 0.0002309	0.99955

[a] Linear regression analysis with a regression equation of $y = ax + b$, in which x is the concentration in ng/ml and y is the relative peak area expressed as % internal standard area (% IS).
[b] Values are mean ± S.D. of three calibrations.
[c] r is the Pearson correlation coefficient obtained from the eight-point calibration curve. The concentrations across the range were evenly distributed.

Table 2
Accuracy, within-day precision (WDP) and between-day precision (BDP) for AZD1152 and hQPA in human plasma, mouse plasma and mouse tissues.

Matrix	AZD1152					hQPA			
	Nominal conc. (ng/ml)	Measured conc. (ng/ml)	Accuracy (%)	WDP (%)	BDP (%)	Measured conc. (ng/ml)	Accuracy (%)	WDP (%)	BDP (%)
Human Plasma	100	99.5	99.5	5.1	8.0	95.1	95.1	7.0	5.2
	1,000	1027.8	102.8	4.6	5.2	1028.7	102.9	3.2	3.4
	10,000	10305.1	103.1	2.6	4.4	10309.1	103.1	1.1	2.3
Mouse Plasma	100	102	102.9	9.32		101	101.7	4.87	
	1,000	999	99.9	3.45		985	98.5	3.67	
	10,000	9,869	98.7	5.65		10,234	102.3	2.28	
Mouse Brain	100	93	93.3	10.29		87	87.1	4.53	
	1,000	925	92.5	2.53		818	81.8	6.11	
	10,000	9,165	91.7	1.82		8,531	85.3	2.00	
Mouse Kidney	100	92	91.9	6.46		88	87.8	5.16	
	1,000	1,128	112.8	5.08		1,077	107.7	2.27	
	10,000	11,039	110.4	0.66		11,055	110.6	1.14	
Mouse Liver	100	90	89.9	2.98		85	85.4	0.13	
	1,000	1,068	106.8	2.21		1,128	112.8	3.83	
	10,000	10,067	100.7	1.38		10,212	102.1	0.97	
Mouse Ileum	100	93	93.2	0.72		85	85.4	6.47	
	1,000	903	90.3	9.92		1,003	100.3	7.10	
	10,000	11,185	111.9	0.62		91,180	91.2	0.17	

Three replicate measurements were performed at each concentration level. Human plasma in four separate analytical runs, mouse plasma and tissues in one run. Within- and between-day precision in human plasma was calculated using one-way analysis of variance (ANOVA) for each control sample concentration level from QCs from fifteen independent HPLC runs.

separate analytical runs. From these results we calculated the accuracy, within-day and between-day precision (Table 2). Next, we determined the accuracy and precision of AZD1152 and hQPA in mouse plasma and a range of tissues also spiked at three levels and analyzed in one analytical run. In all cases the accuracy and precision was well within the limits that are considered acceptable for bioanalytical assays.

The assay was subsequently used for the bioanalysis of study specimens and with each run ($n = 15$) a set of quality control specimens low, mid and high were analyzed in duplicate. The accuracy of the grand mean was for both AZD1152 and hQPA within ±5% of the nominal value, whereas the highest WDP% and BDP% were 8.3% and 10.6%, which were observed for hQPA in the 100 ng/ml quality control specimen.

The recovery of AZD1152, hQPA and MTX spiked at 100 and 10,000 ng/ml in human plasma, mouse plasma and tissues was acceptable within the 84–99% range (Table 3).

3.6. Inhibition of phosphatase activity in mouse plasma and liver homogenate

In order to determine the phosphatase activity and to establish the most optimal conditions to inhibit this phosphatase activity in order to minimize *ex vivo* conversion of AZD1152 into hQPA, mouse plasma spiked with AZD1152 was incubated under four different conditions (Fig. 4). As expected the phosphatase activity was substantial at ambient temperature with more than 20% of the AZD1152 being recovered as hQPA after 8 h of incubation. The addition of NaF and EDTA inhibited phosphatase activity but mostly at later time points (8 and 24 h of incubation; $P = 0.18$ and 0.0013, respectively). The lowest phosphatase activity was observed at 0 °C, and further addition of NaF and EDTA contributed only significantly ($P = 0.023$) after 24 h. In order, to test whether this latter condition is also suited to reduce the phosphatase activity in mouse tissue homogenates, liver homogenates from three different mice were spiked with AZD1152 and incubated at 0 °C, ambient temperature and/or in the absence or presence of NaF and EDTA for 0, 0.25, 0.5, 1, 4, 8, and 24 h. The phosphatase activity in these liver homogenates under both temperature conditions was comparable to plasma. In all cases, the conversion of AZD1152 to hQPA was quantitative and no other peaks were observed in the chromatograms. Based on these data we conclude that biological samples must be cooled to 0 °C immediately after collection and subsequently processed (homogenization and ethanol precipitation) within 1 h. Although the effect of NaF and EDTA on dephosphorylation of AZD1152, compared to cooling was small, we do recommend the use of these additives.

3.7. Sample stability

The stability of primary, secondary, and internal standard stock solutions is at least 1 year at −20 °C (data not shown). Unprocessed human plasma samples spiked with AZD1152 and hQPA at 100 and 10,000 ng/ml were unstable when stored at −20 °C. The AZD1152 in these samples was progressively dephosphorylated to

Table 3
Extraction recovery of AZD1152, hQPA and MTX from human plasma, mouse plasma and mouse tissues, each from three different subjects.

Matrix	Nominal conc.[a] (ng/ml)	Recovery ± C.V. (%)		
		AZD1152	hQPA	MTX
Human Plasma	100	95 ± 4.5	93 ± 5.6	98 ± 4.7
	10,000	98 ± 4.7	97 ± 3.3	99 ± 2.9
Mouse Plasma	100	94 ± 5.5	92 ± 6.1	99 ± 2.6
	10,000	92 ± 5.8	91 ± 4.3	98 ± 4.8
Mouse Brain	100	89 ± 6.7	84 ± 4.0	93 ± 2.3
	10,000	91 ± 3.4	88 ± 4.1	94 ± 3.7
Mouse Kidney	100	90 ± 5.6	89 ± 3.0	94 ± 6.0
	10,000	92 ± 7.1	89 ± 2.4	93 ± 1.9
Mouse Liver	100	89 ± 4.9	87 ± 3.5	95 ± 2.8
	10,000	92 ± 3.8	89 ± 5.2	96 ± 6.7
Mouse Ileum	100	91 ± 4.4	90 ± 5.7	95 ± 4.4
	10,000	95 ± 5.0	92 ± 4.2	95 ± 3.2

Three replicate measurements were performed for mouse plasma and tissues at each concentration level.

$$\text{C.V. (coefficient of variation)} = \left(\frac{\text{S.D.}}{\text{mean}}\right) \times 100.$$

[a] The nominal concentration represents the low, and high quality control concentrations lying within the standard curve.

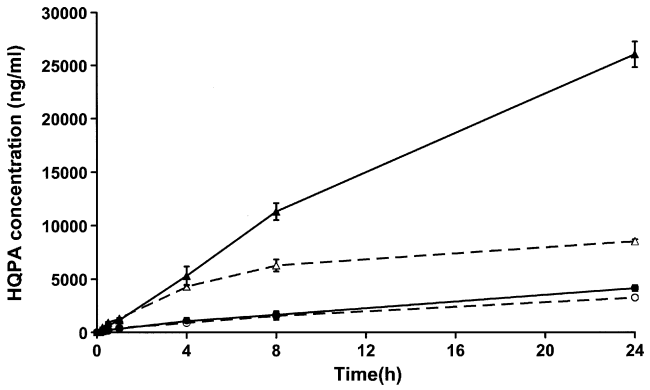

Fig. 4. Inhibition of phosphatase activity in mouse plasma spiked with AZD1152 at 50,000 ng/ml and incubated at four different conditions: (▲) 37 °C without inhibitors; (△) 37 °C with NaF and EDTA; (●) 0 °C without inhibitors; (○) 0 °C with NaF and EDTA. At indicated times, dephosphorylation was stopped by addition of ethanol and hQPA was determined by HPLC analysis. Data are expressed as means ± S.D. of three different samples.

hQPA resulting in 15 ± 6% and 27 ± 5% decrease of AZD1152 after 24 and 48 h, respectively. Processed human plasma samples spiked with AZD1152 and hQPA at 100 and 10,000 ng/ml were stable in the autosampler for at least 48 h. During this time period measured levels of AZD1152, hQPA and MTX deviated less than 7.4%, 6.5% and 4.9% from the nominal concentrations, respectively. Similarly, spiked and processed samples had acceptable long term storage stability at −20 °C. After 3 months storage under these conditions, the measured concentrations deviated by less than 4.7% and 4.4% from the nominal spiked concentrations of 100 and 10,000 ng/ml, respectively.

3.8. Applicability of method for determination of pharmacokinetics in wild-type FVB mice

To demonstrate the practical applicability of the method, nine female wild-type FVB mice were dosed with AZD1152 as described in materials and methods. Plasma levels of AZD1152 and hQPA were about 4 times the LLOQ at 8 h and 24 h, respectively. At earlier time points the concentrations of AZD1152 and hQPA were up to 200 times higher, thus allowing easy quantification (Fig. 5A). The levels of AZD1152 and HQPA in brain, kidney, and liver, were determined after 2, 6, and 24 h, and levels in the ileum were determined after 6 h (Fig. 5B). In these tissues, the concentrations of AZD1152 were below LLOQ at all time points, thus indicating rapid *in vivo* conversion into hQPA. The concentration of hQPA in brain was approximately 150 ng/g at 24 h; concentrations in the ileum were 446 ng/g at 6 h post dose. In the liver and kidney, hQPA levels were lowest at 24 h, but still 20–60 times above the LLOQ.

4. Discussion

Currently, no analytical method has been published for the determination of AZD1152 or hQPA in biological samples. In the present paper we describe the development and validation of a HPLC based assay with UV detection for the simultaneous determination of AZD1152 and hQPA in human plasma, mouse plasma and tissues.

At the outset, the major issue with setting up an assay to measure both AZD1152 and HQPA related to instability of the prodrug in biological material such as plasma, and tissues. This is due to the vulnerability of the phosphate prodrug AZD1152 to dephosphorylation by phosphatase enzymes in the various tissues and plasma samples. In order to minimize *ex vivo* conversion of AZD1152, EDTA and NaF should be added to plasma and tissue homogenate samples and cooled to 0 °C immediately. Subsequently, sample preparation using ethanol precipitation of proteins should be performed within 1 h, as this step efficiently annihilates residual phosphatase activity. Another complication we encountered during the development of the assay was the large difference in polarity between AZD1152 and hQPA. In order to minimize this polarity difference we selected a pH 3.4 for the mobile phase. At this pH the compounds have only moderate differences in hydrophobicity making the separation of both compounds in a single HPLC run feasible. Complete separation of AZD1152 and hQPA was established within 17 min using a simple isocratic mobile phase containing 50 mM phosphate buffered water and 18.5% acetonitrile. Because of endogenous interferences eluting at later elution times, the HPLC run time was extended to 30 min. In our experience this gave satisfactory results and is more convenient than using a mobile phase gradient with higher acetonitrile content. The applied Uptisphere C18 HDO HPLC column is specially designed for the separation of mixtures containing compounds with moderate differences in hydrophobicity. Another big advantage of the Uptisphere HDO column is the almost complete absence of tailing due to interactions between aromatic ring structures in the analytes and residual silanols. However, some tailing of AZD1152 was observed which we believe may be due to interactions between the phosphate group on the AZD1152 and the stationary phase. In this assay we used a phosphate buffered mobile phase, which prevented unwanted interactions between the phosphate prodrug and the silica particles. Although the extraction with

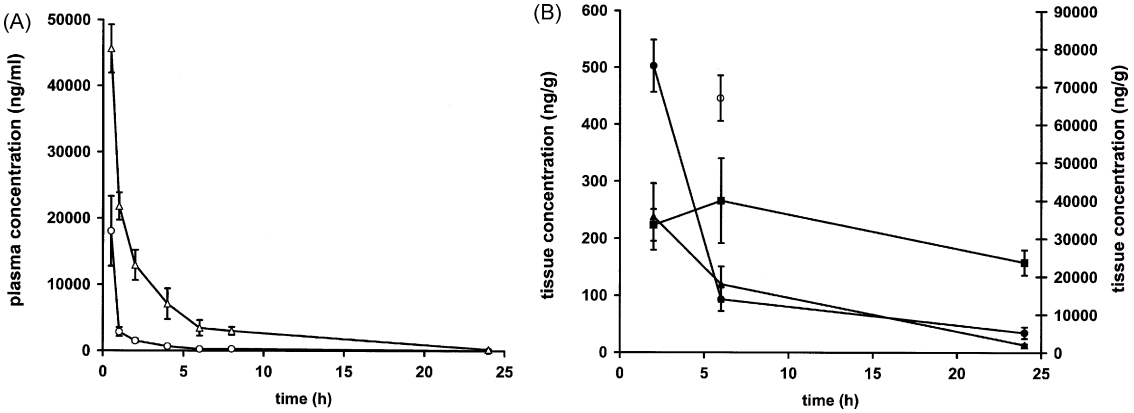

Fig. 5. Levels of AZD1152 (○) and hQPA (△) in plasma (A), and hQPA levels in liver, kidney and brain (B), from mice intra-peritoneal dosed with a single bolus of 100 mg/kg AZD1152. Brain (■) and ileum (○) hQPA levels are represented on the left *y*-axis, liver (▲) and kidney (●) levels are on the right axis. Data are expressed as means ± S.D. of three different mice.

ethanol provided a sample suitable for analysis we did observe some deterioration of the hQPA peak shape. However, this was resolved by replacing the guard column after about 50–100 samples.

The assay was first validated using human plasma as biological matrix as this is more readily available in large quantities than mouse plasma and tissues. By analyzing the calibration curves we were able to show that the assay has a linear response for both compounds over the complete concentration range tested from 25 up to 10,000 ng/ml. The accuracy lies within a range of 85–115% for both compounds, which is acceptable. Also the within-run precision of 11% and between-run precision of 8.5% are acceptable. Although addition of MTX as an internal standard has no merit for the accuracy and precision of the assay, we do believe it increases the robustness of the assay. The LLOQ of the assay was 25 ng/ml for AZD1152 and hQPA. At the LLOQ the assay variability was within acceptable levels, with at least 80% of the samples deviating less than 20% from the nominal concentrations. Because the absolute recovery of AZD1152 and hQPA in mouse plasma and tissue homogenates was not different from human plasma this latter matrix was used for preparation of calibration curves for measurement in all mouse matrices. The accuracy and precision of AZD1152 and hQPA in quality control specimens prepared in the various mouse matrices was always within the acceptable limits.

Stability of AZD1152, hQPA and MTX in processed samples in the autosampler was acceptable, with no detectable degradation after 48 h, at all time points measured concentrations showed less than 10% deviation from the nominal concentration. Furthermore, AZD1152 and hQPA were shown to be stable in processed human plasma samples when stored at −20 °C for 3 months.

In conclusion, an assay has been developed and validated to determine the concentrations of AZD1152 and hQPA in human plasma, mouse plasma and tissues. The assay is straightforward in terms of sample preparation, uses isocratic elution and UV detection. It is currently being used for determining AZD1152 and hQPA concentrations in mouse plasma and tissues from FVB wild-type and ABC-drug transporter knock out mice. We expect that the method may also be suitable for determination of AZD1152 and hQPA in other animal or human tissues, cell lines or culture media, but this will require additional validation.

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