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Liquid chromatography–tandem mass spectrometric assay for the simultaneous determination of the irreversible BTK inhibitor ibrutinib and its dihydrodiol-metabolite in plasma and its application in mouse pharmacokinetic studies

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ABSTRACT

A validated simple, fast and sensitive bio-analytical assay for ibrutinib and its dihydrodiol metabolite in human and mouse plasma was set up. Sample preparation was performed by protein precipitation, and addition of the respective deuterated internal standards, followed by LC–MS/MS analysis. Separation was performed on a 3.5 μm particle-size, bridged ethylene hybrid column with gradient elution by 0.1% v/v formic acid and acetonitrile. The full eluate was transferred to an electrospray interface in positive ionization mode, and subsequently analyzed by a triple quadrupole mass spectrometer by selected reaction monitoring. The assay was validated in a 5–5000 ng/ml calibration range. Both ibrutinib and dihydrodiol-ibrutinib were deemed stable under refrigerated or frozen storage conditions. At room temperature, ibrutinib showed a not earlier described instability, and revealed rapid degradation at 37 °C. Finally, the assay was used for a pharmacokinetic study of plasma levels in treated FVB mice.

1. Introduction

Ibrutinib is an orally administered tyrosine kinase inhibitor (TKI) for the treatment of B-cell malignancies. It was approved by the US FDA in November 2014 [1] and by the EMA as an orphan medicinal product in 2012 for treatment of mantle cell lymphoma (MCL), in 2013 for treatment of chronic lymphocytic leukemia (CLL), and in 2015 as the first registered drug for Waldenström macroglobulinemia (WM). The European Committee for Medicinal Products for Human Use (CHMP) adopted a positive opinion, recommending the granting of an initial marketing authorization in 2014 [2,3]. Ibrutinib covalently binds and inhibits the Bruton's tyrosine kinase (BTK) protein. BTK is a signaling molecule of the B-cell antigen receptor (BCR) and cytokine receptor pathways [4]. Since the market authorization, several research articles have been published on the use of Ibrutinib for diseases other than B cell malignancies, such as overactive immune response related (rheumatic) diseases [5] and even more possible drug-targets have been described (MRP1, ErbB2) [6,7]. Pharmacokinetic evaluations of ibrutinib, however, are still scarce. In the first pharmacokinetic studies there was very limited information concerning the quantitative bio-analytical method [8–11]. Since then, some studies have been completed using more detailed methods, although they do not analyze the main metabolite or do not make use of a satisfying internal standard. In addition, they use long run times, high sample volume and laborious sample preparation methods [12,13]. Although one method has been described analyzing ibrutinib in dog, rat and monkey plasma (but

Abbreviations: BCR, B-cell antigen receptor; BSA, bovine serum albumin; BTK, Bruton's tyrosine kinase; CHMP, Committee for Medicinal Products for Human Use; DHI, dhihydrodiol-ibrutinib; DMSO, dimethyl Sulphoxide; EMA, European Medicines Agency; ErbB2, receptor tyrosine-protein kinase erbB-2; ESI, electro spray ionization; FDA, Food and Drug Administration; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LLOQ, lower limit of quantitation; MRP1, multidrug resistance protein 1; PP, polypropylene; QC, quality control; SRM, selective reaction monitoring; ULOQ, upper limit of quantitation; WM, Waldenström macroglobulinemia.

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not the metabolite) [13], none have been described for the analysis in mouse plasma. Therefore, the development and validation of a simple, fast and sensitive bio-analytical assay for ibrutinib and the dihydrodiol metabolite in human and mouse plasma was set up. This method uses a simple protein precipitation step, followed by LC–MS/MS analysis. This assay proves a useful tool in pharmacokinetic studies pertaining to ibrutinib, and was subsequently used for the evaluation of plasma levels in mice and in the assessment of the not earlier described breakdown of ibrutinib in human plasma.

2. Materials and methods

2.1. Chemicals and reagents

Ibrutinib (>99.9%, mw: 440.51), the main metabolite dihydrodiol-ibrutinib (95.9%, mw: 474.52) and the respective [$^2H_5$]-labeled ibrutinib (acetate salt, >99.9%, mw: 445.54), and dihydrodiol-ibrutinib (>99.9%, mw: 479.23) were obtained from Alsachim (Illkirch Graffenstaden, France). LC–MS grade water, methanol of HPLC quality and acetonitrile of HPLC-S gradient grade quality were acquired from Biosolve (Valkenswaard, The Netherlands). Water not used as eluent was home-purified by reversed osmosis on a multi-laboratory scale. Formic acid was of analytical grade and originated from Merck (Darmstadt, Germany) and analytical grade dimethyl sulfoxide (DMSO) was supplied by Acros (Geel, Belgium). Human and mouse lithium-heparinized plasma (pooled human and mouse, and from individual mice) were obtained from Sera Laboratories International (Haywards Heath, West Sussex, UK). For the evaluation of selectivity and matrix effect, human lithium-heparinized plasma of six individual donors were obtained from Innovative Research (Novi, MI, USA).

2.2. Chromatography and MS/MS method

The LC-system consisted of a DGU-14A degasser, a CTO-10Avp column oven, a Sil-HTc auto sampler and two LC10-ADvp-μ pumps (all from Shimadzu, Kyoto, Japan). 5 μl of the plasma extract was injected on a Waters XBridge BEH300 column (2.1 × 50 mm, dp = 3.5 μm, Waters, Milford, USA), protected by a Agilent Polaris3C18-A MetaGuard pre-column (10 × 2.0 mm, dp = 3.0 μm, Agilent, Santa Clara, USA). The column temperature was maintained at 40 °C and the auto injector at 4 °C. Elution was performed by a gradient of (**A**) 0.1% (v/v) formic acid in water and (**B**) methanol at a flow of 0.5 ml/min. The gradient started at 40% methanol, increasing linearly to 85% methanol in 2.0 min, after which an isocratic plateau was held for 0.5 min, till 2.5 min. At 2.5 min, the level of methanol was decreased back to 40%, at which the column was equilibrated for 0.5 min until the following injection. The whole eluate was transferred into the electrospray probe, starting at 1.2 min after injection by switching the MS inlet valve, until 2.4 min of the 3 min analytical run. For detection of all compounds, a TSQ Quantum Discovery Max quadrupole mass spectrometer with electrospray ionization (Thermo Fisher Scientific, San Jose, CA, USA) was used. Data were recorded on, and the system was controlled by the Thermo Fisher Xcalibur software (Version 2.0.7 SP1). The electrospray was tuned in the positive ionization mode by introducing 0.5 ml/min of a mixture of Methanol and 0.1% v/v formic acid in water (50/50, v/v), while infusing 5 μg/ml ibrutinib or dihydrodiol-ibrutinib at 5 μl/min. The highest response was obtained with a 5000 V spray voltage, a 400 °C capillary temperature and the nitrogen sheath, ion sweep and auxiliary gasses set at 50, 0 and 5 arbitrary units, respectively; the skimmer voltage was set off (0 V). The SRM mode was used with argon as the collision gas at 1.5 mTorr. The tube lens off-set was 103 V for both compounds. Ibrutinib was monitored at m/z 441.2 → 304.2 and 84.1 at −27 and −33 V collision energies and 0.100 s dwell time, the dihydrodiol-metabolite at m/z 475.2 → 304.2 and 84.1 at −27 and −33 V collision energies and 0.100 s dwell time. The mass resolutions were set at 0.7 for both separating quadrupoles.

2.2.1. Qualitative analysis

To chromatographically separate unknown metabolites of ibrutinib, a slower gradient method was set up. Ten microliter was injected onto column, followed by a 15 min gradient, starting at 5% methanol, increasing linearly to 100% methanol in 13 min, after which an isocratic plateau was held for 1 min, till 14 min. At 14 min, the level of methanol was decreased back to 5%, at which the column was equilibrated for 1 min until the following injection. For detection of any unknown metabolites, various scanning methods were used for MS and MS/MS: the aforementioned SRM, a parent ion scan of product m/z 84.1, neutral loss scan of m/z 84.1 and full ESI scans ranging between m/z 50–650 (coarse) and 200–500 (fine).

2.2.2. Data processing

For chromatographic data, Thermo Fisher Xcalibur software (Version 2.0.7 SP1) was used. For further data processing Microsoft Excel (Office 2010, Version 14.0.7147.5001) and GraphPad Prism 6 (Version 6.05) were used. Calculations of the pharmacokinetic data were made using the PK-solver add-in for Excel [14]. Averages (±SD) were calculated using MS Excel.

2.3. Standard solutions and quality controls

Stock solutions of 500,000 ng/ml were made by weighing 300–500 μg, and dissolving this in 600–1000 μl methanol (ibrutinib) or DMSO (dihydrodiol-ibrutinib, and both internal standards). Dual calibration standards were prepared from a 5000 ng/ml standard solution in human plasma, and stored in aliquots at −30 °C until further use. For this, the 5000 ng/ml standard solution was diluted to produce the calibration curve of 5000, 2000, 500, 200, 50, 20 and 5 ng/ml using blank human plasma.

Quality control (QC) samples were prepared from separate 500,000 ng/ml stock solutions. The 500,000 ng/ml solutions were diluted using blank plasma to produce QCs of 4000 (high), 400 (medium), 15 (low) and 5 (lower limit of quantitation, LLOQ) ng/ml in blank human or mouse plasma. QCs were stored in a similar fashion to calibration samples.

2.4. Sample preparation

Plasma samples were pretreated by protein precipitation. For all matrices, in a polypropylene (PP) reaction tube, 30 μl 150 ng/ml internal standard solution in acetonitrile was added to 20 μl plasma, followed by vortex mixing for approximately 5 s. The samples were centrifuged at 15,000 × g for 5 min at 15 °C. Fourty microliter of the supernatant was transferred to a 1.5 ml vial with a 250 μl glass micro-insert. Before closing the vial, 40 μl of 50% v/v methanol was added. For all samples, 5 μl sample was injected onto the column.

2.5. Degradation study of ibrutinib in human plasma

Degradation of ibrutinib was investigated in human and murine plasma. To exclude the influence of anticoagulant, multiple anticoagulants were tested, namely lithium-heparin, sodium-EDTA and the absence of one by testing serum, as EDTA can have a possible effect on catalysis by capturing metal ions [15,16]. Samples of 5000 ng/ml in each matrix were subjected to incubation at 37 °C, and aliquots were taken at 0, 3 and 18 h.

For the determination of the conversion rate, blank human serum was used. Serum was chosen over plasma for the above

mentioned reasons, and as a control 70 g/l heat inactivated bovine serum albumin (BSA) was used. BSA was to prevent ibrutinib from precipitating. The conversion rate was calculated by the integration of rate = $-d[A]/dt$ from time zero to 6 h in spiked human serum ($[A]_0$: 5000 ng/ml, or 11.35 μM). The samples were incubated at 37 °C, and samples were taken at 0, 1, 2, 4, 6, and 12 h.

For the elucidation of the unknown degradation product, the degraded sample was compared against an aliquot of the same sample directly stored at −30 °C, using the 15 min chromatographic method. These samples were pre-treated by adding 100 μl acetonitrile to 50 μl plasma or serum. Samples were diluted 1:1 by adding 50% v/v methanol.

2.6. Analytical method validation

A laboratory scheme based on international guidelines, published by the EMA [17] and FDA [18] was used for the validation procedures.

2.6.1. Calibration

Calibration solutions of ibrutinib and dihydrodiol-ibrutinib were prepared by diluting stock solutions of 500,000 ng/ml to 5000 ng/ml in human lithium heparin plasma. These solutions were stored until further use in aliquots in 1.5 ml polypropylene tubes. The calibration samples were prepared from these solutions as described in Section 2.3, and were processed in duplicate for each daily calibration. Least squares linear regression was used to define the calibration curve, using the analyte/IS peak ratio.

2.6.2. Precision and accuracy

Separate stock solutions of 500,000 ng/ml of ibrutinib and dihydrodiol-ibrutinib were used to obtain validation samples in human plasma (4000, 400, 15, 5 ng/ml). Precision and accuracy were determined by sextuple analysis of each QC in three analytical runs on three separate days for all QCs (total: $n = 18$ per QC). Relative standard deviations were calculated for both, the within- and between-day precisions.

For the mouse plasma, partial validation was performed. For this, precision and accuracy were determined by sextuple analysis of each QC, along with matrix effect and selectivity. This partial validation is in accordance with the FDA guidelines [18].

2.6.3. Selectivity

To assess the selectivity, six individual human plasma and mouse samples were processed to test the selectivity of the assay. Samples were processed without ibrutinib and dihydrodiol-ibrutinib and IS and with ibrutinib and dihydrodiol-ibrutinib at the LLOQ level (5 ng/ml), supplemented with the IS.

2.6.4. Recovery and matrix effect

The recovery was determined ($n = 6$) by comparing processed samples (QC-high, -med, -low) with reference solutions of ibrutinib and dihydrodiol-ibrutinib in blank plasma extract at the same levels. To assess the matrix effect in human plasma, the reference solutions in blank plasma extracts were compared to matrix-free solutions at the three same levels. For murine samples, an experiment was performed in which ibrutinib, dihydrodiol-ibrutinib and their internal standards were continuously infused (5 μg/ml, 5 μl/min) during a run, in which blank extracts (plasma:acetonitrile, 1:1.5) were injected on the column.

Recovery of ibrutinib, dihydrodiol-ibrutinib and their respective deuterated internal standards was determined by comparing the response of ibrutinib and dihydrodiol-ibrutinib in an extracted plasma sample to spiked blank extract.

2.6.5. Stability

The stabilities of ibrutinib and dihydrodiol-ibrutinib was investigated in QC-high and -low plasma samples stored in polypropylene tubes. Quadruplicate analysis of human and mouse samples from separate tubes was performed after storage at 0 °C for 8 h, followed by three freeze-thaw cycles (thawing at 0 °C, during ca. 6 h and freezing again at −30 °C for at least 12 h). Furthermore, an analytical run was re-injected after additional storage of the extracts at 4 °C for 24 h to test the stability at the conditions in the auto injector. Finally, the responses of ibrutinib and dihydrodiol-ibrutinib from the stock solutions in methanol and DMSO, respectively, after 2 months at −30 °C ($n = 2$) were compared to fresh stock solutions with LC–MS/MS after appropriate dilution of the samples with 50% v/v methanol, and adding internal standards.

2.7. Pharmacokinetic study in mice

For the pilot pharmacokinetic study in mice, five wild-type Friend leukemia virus B (FVB) mice were given ibrutinib (10 mg/kg) by gavage, and subsequently, 50 μl blood samples were drawn at 5, 15, 30, 60 and 180 min from the tail vein. The animals were terminated at 480 min, at which the final blood sampling was done via heart-puncture. All samples were directly placed on ice after sampling, and were stored at −30 °C. Animals were housed and handled according to institutional guidelines in compliance with Dutch and European legislation. All experiments were approved by the Institutional Animal Care and Use Committee of the Netherlands Cancer Institute.

3. Results and discussion

3.1. Method development

3.1.1. Chromatography and LC–MS/MS method

Positive ESI-MS/MS settings were optimized for protonated ibrutinib (m/z 441.2), dihydrodiol-ibrutinib (475.2), and their deuterated internal standards (446.2 and 480.2 respectively). For maximal sensitivity the sums of the two most prominent dissociation products for each compound were measured (ibrutinib and dihydrodiol-ibrutinib: m/z 304.1 and 84.1, deuterated internal standards: 309.1 and 84.1). Product spectra of ibrutinib and dihydrodiol-ibrutinib are shown in Fig. 2; dissociation products at m/z 304.1 have been used in an existing assay [12]. The chromatographic method was optimized empirically, based on MS response, retention time, and peak shape. Methanol, in combination with 0.1% formic acid gave best results in chromatography. With a total run-time of 3 min, the components eluted at 1.6 min (dihydrodiol-ibrutinib) and 1.9 min (ibrutinib). Representative chromatograms of extracted human plasma, with and without analyte and/or IS are shown in Fig. 1. Retention-times of the internal standards matched those of the native compounds. Based on retention time stability (RSD <1% for both compounds), column equilibration of 0.5 min showed sufficient equilibration. Time between analytical runs was around 30 sec, resulting in additional equilibration of the column.

3.1.2. Qualitative analysis

The 15 min method gave satisfying separation. The retention time of ibrutinib was 8.3 min, the unknown degradation product eluted at 10.4 min, and showed the most prominent response in the neutral loss scan of m/z 84.1.

3.1.3. Sample preparation

In the analysis of small-molecule kinase inhibitors, LC–MS/MS, following protein precipitation, has often been applied in sensitive bioanalytical assays in multiple matrices [19,20]. This method

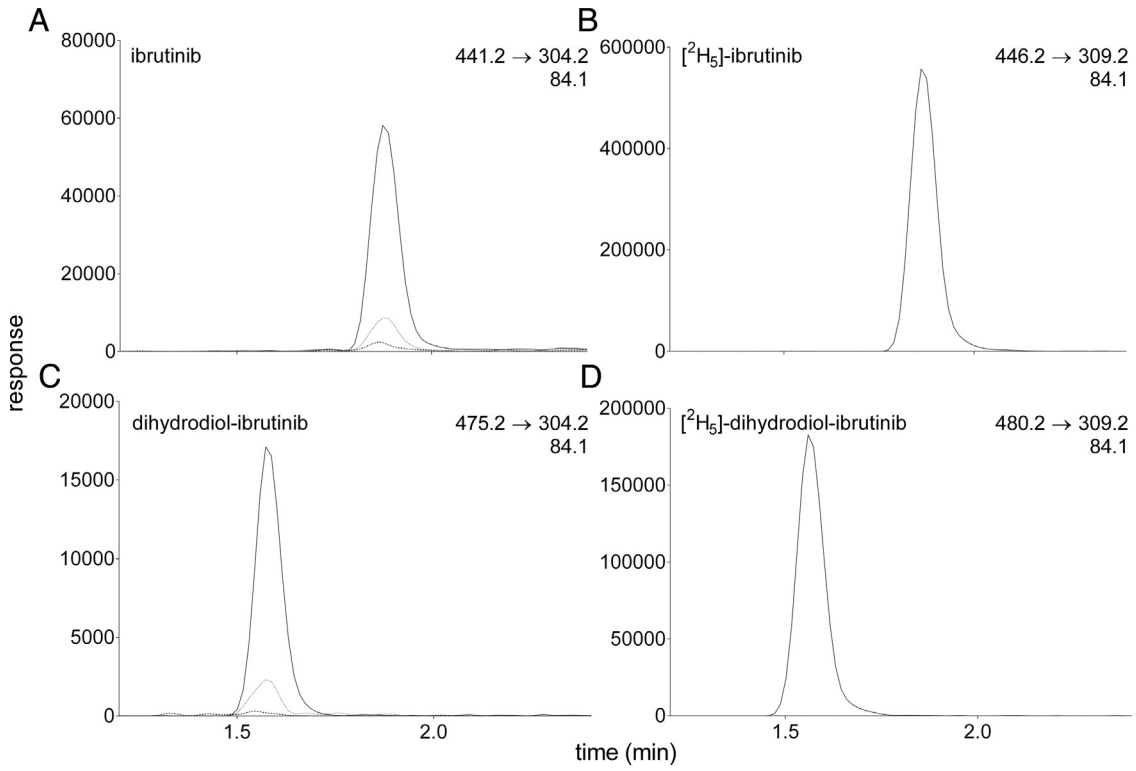

Fig. 1. Chromatograms of ibrutinib (A), [$^2H_5$]-ibrutinib (B), dihydrodiol-ibrutinib (C), [$^2H_5$]-dihydrodiol-ibrutinib (D) in human plasma at 5 ng/ml (dihydrodiol-) ibrutinib, with 150 ng/ml [$^2H_5$]-(dihydrodiol-) ibrutinib added (—), blank human plasma, with 150 ng/ml [$^2H_5$]-(dihydrodiol-) ibrutinib added (· · ·) or plain blank human plasma (- - -).

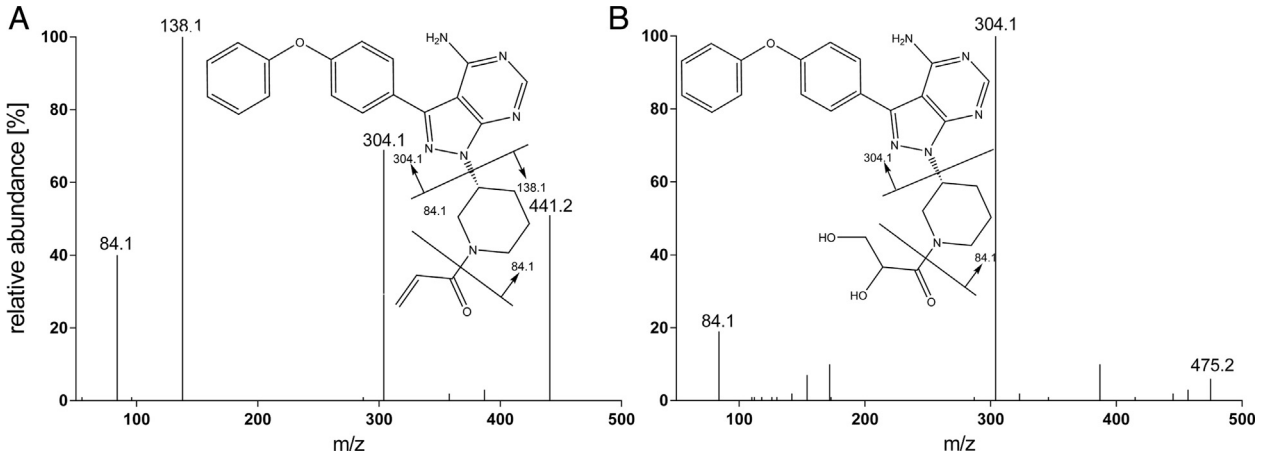

Fig. 2. Fragmentation pattern of (A) ibrutinib and (B) dihydrodiol-ibrutinib at 1.5 mTorr collision gas, and 25 V collision energy.

is less labor intensive than the methods that use liquid–liquid extraction [12], or even solid phase extraction [21]. As organic precipitation agent for plasma, acetonitrile shows the most efficient precipitation of plasma proteins, as shown by Blanchard [22]. Meanwhile, the amount of precipitating agent was kept as small as possible (1.5:1) to limit sample dilution.

3.1.4. Degradation study of ibrutinib in human plasma

During the development of this assay, stability of ibrutinib at room temperature seemed to be an issue. Therefore, a short study was performed to elucidate the potential mechanism behind this degradation. Two articles on the analysis of ibrutinib in plasma did not report the degradation of the compound in plasma [12,13]. We found, however, that ibrutinib is relatively stable in murine plasma, but it rapidly degrades in human plasma at 37 °C, or even room temperature (Table 1).

Table 1
Change in ibrutinib-concentration at ambient (bench-top) or elevated temperatures. Data is represented as average ± SD.

Species	Temperature (°C)	Time (h)	Compound	Change (%)	(*n*)
Human	22	3	Ibrutinib	−4.3 ± 4.5	4
	22	3	DHI	−0.9 ± 1.9	4
	22	12	Ibrutinib	−47.2 ± 2.4	3
	22	12	DHI	2.9 ± 7.3	3
	37	3	Ibrutinib	−20.5 ± 0.7	3
Mouse	37	3	Ibrutinib	3.4 ± 3.2	2

Since ibrutinib showed considerable break-down when stored at room temperature in lithium-heparin plasma, up to 50% overnight, this effect was further investigated. The effect looked

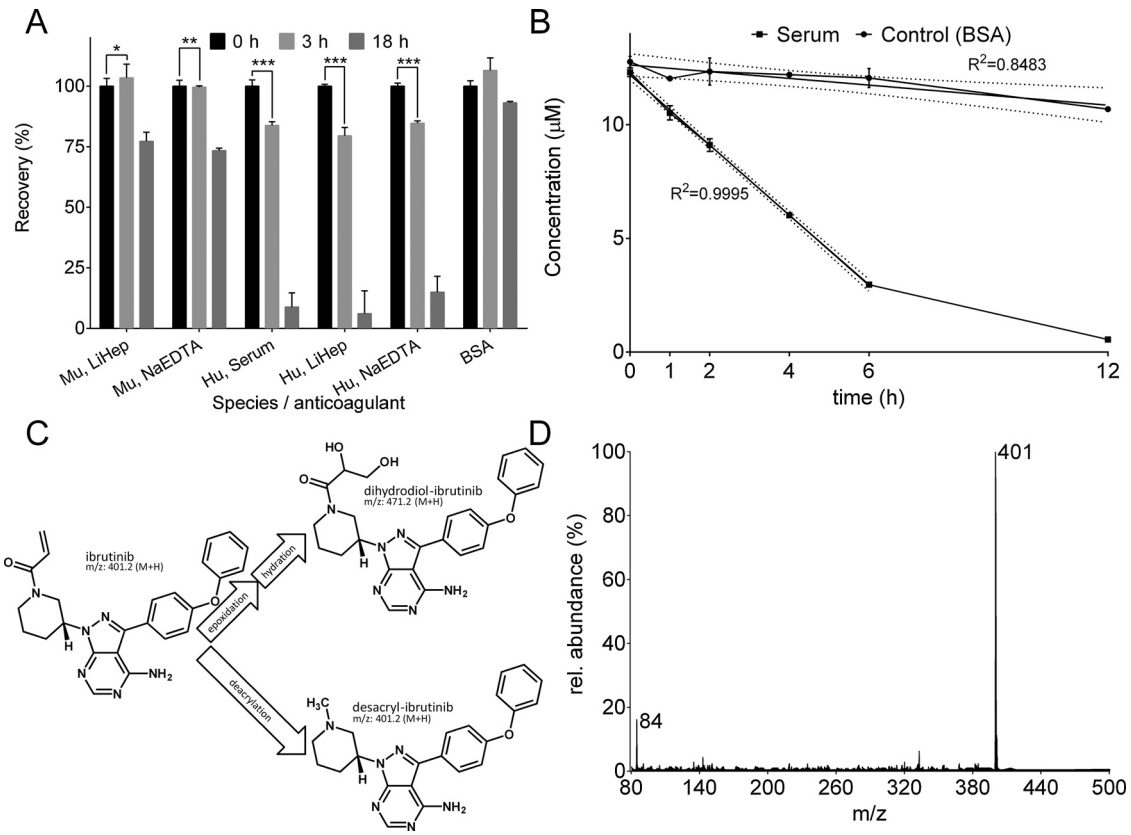

Fig. 3. (A) Metabolism of Ibrutinib at 37 °C in mouse and human plasma, human serum, and BSA using different anticoagulants. (B) Concentration versus time curve of ibrutinib in serum (and control) shows a zero-order catalyzed reaction (Mu: Murine; Hu: Human; LiHep: blood-plasma, lithium-heparin anticoagulated; NaEDTA: blood plasma, sodium-ethylenediaminetetraacetic acid anticoagulated; BSA: bovine serum albumin) *P 0.54, **P 0.79, ***P < 0.001. (C) Metabolic scheme of ibrutinib to dihydrodiol-ibrutinib and the proposed degradation product desacryl-ibrutinib. The degradation product is only found in-vitro in human plasma. (D) Product spectrum of desacryl-ibrutinib, acquired during a prolonged chromatographic run with a 25 V collision energy.

like a temperature-dependent reaction, given that the break-down is almost entirely stopped by storing at 4 °C (data not shown). Given this fact, an experiment was set up to investigate the degradation at 37 °C. Heat-inactivated albumin at physiological concentration was used as a control. Murine plasma showed results not much different to the BSA controls, so possibly only non-catalyzed breakdown at 37 °C was shown, as there is a slight decrease in ibrutinib in these as well (Fig. 3A).

The degradation of ibrutinib was studied in various plasma samples. For the determination of the reaction rate coefficient, human serum was used. This was chosen for the absence of anti-coagulants, such as EDTA, which can have a possible inhibitory effect on enzymatically catalyzed reactions [15,16]. However, the effect of anticoagulants on drug stability in matrix is refuted in one article [23]. The concentration versus time curve shows a zero-order catalyzed reaction, and the reaction rate at 37 °C was determined at 1.543 ± 0.207 nM/h (95% CI:1.477–1.609) versus 0.147 ± 0.031 nM/h (95CI: 0.061–0.234) in control samples (Fig. 3B). Sodium EDTA and lithium heparin showed similar results (Fig. 3A).

An effort was made to identify the reaction product. A new product of m/z 401.2 was found in the samples, which were incubated for 12 h. This product is most likely due to the loss of the acrylmoiety of the reactive acrylamide group, and thus rendering the compound unable to covalently bind BTK [4,13]. This is supported by the fact that the dihydrodiol-metabolite does not undergo the same degradative fate as the parent compound (see stability data in Section 3.2.5). Although this reaction occurs to a much lesser extent than the hepatic metabolism of ibrutinib, the break-down still continues after blood is drawn. For this reason our protocol dictates working on ice directly after drawing blood, during handling, also thawing should be done on ice, and storage at −30 °C, to prevent degradation as much as possible. The stability issue could prove to be an issue in therapeutic drug monitoring, neglected in clinical studies so far, based on current literature. After blood sampling, the blood might be directly centrifuged to separate cells and plasma, but will most likely not be analyzed directly.

3.2. Analytical method validation

In our assay, an upper limit of quantitation (ULOQ) was proposed at 5000 ng/ml. This level was chosen to accommodate for the possibility of outliers in case of slow metabolic elimination or enhanced uptake due to inhibition of first pass metabolism, as the original research indicated a vast variation in plasma levels of the drug and its metabolite [9]. Following the here gathered pharmacokinetic data (Section 3.3), a lower LLOQ could provide some extra information, although levels below 5 ng/ml would not be likely to prove relevant in-vivo.

3.2.1. Calibration

The calibration curves were linear over the ranges examined, with a coefficient of determination (R^2) greater than 0.998. Since the calibration curves of both compounds did not show non-linearity, a linear-regression model using least squares regression (quadratic regression) was used. The linear functions were $Y = (0.013 \pm 0.005) \times X + (0.005 \pm 0.001)$ for ibrutinib and $Y = (0.013 \pm 0.006) \times X + (0.016 \pm 0.004)$ for the dihydrodiol metabolite (mean ± SD). Where X is the ratio of the analyte response

Table 2
Within and between day precision, and accuracy of ibrutinib and dihydrodiol-ibrutinib (DHI) in spiked human plasma samples ($n = 18$).

Level (ng/ml)	Compound	Within day precision (%)	Between day precision (%)	Accuracy (%)
4000	Ibrutinib	1.6	5.6	99.9
	DHI	4.1	5	101
400	Ibrutinib	4.1	6.5	97.6
	DHI	3.6	7.9	102.9
15	Ibrutinib	3.6	6.9	106.2
	DHI	8.8	9.4	99.7
5	Ibrutinib	10.1	11.1	111.2
	DHI	10.5	11.7	97.4

Table 3
Within day precision, and accuracy of ibrutinib and dihydrodiol-ibrutinib (DHI) in spiked mouse plasma samples ($n = 18$).

Level (ng/ml)	Compound	Within day precision (%)	Between day precision (%)	Accuracy (%)
4000	Ibrutinib	2.2	4.7	92.3
	DHI	2.4	3.6	96.5
400	Ibrutinib	2.4	3.1	97.4
	DHI	2.6	4.8	99.1
15	Ibrutinib	4.5	5.5	99.1
	DHI	3.4	4.3	105.2
5	Ibrutinib	11.9	12.0	99.4
	DHI	10.7	12.8	101.0

versus the internal standard response, and Y the concentration in ng/ml.

3.2.2. Precision and accuracy

In Tables 2 and 3, the accuracy and precision of the method in human plasma and mouse plasma are shown. The precision and accuracy for three analytical runs for human, and one for mouse, were within the 100 ± 15% for high, medium, and low QC's, and 100 ± 20% for the LLOQ QC's, as required [17,18].

3.2.3. Selectivity

We have shown that along with chromatographic separation, any remaining endogenous substance does not co-elute with ibrutinib and/or dihydrodiol-ibrutinib, and thus does not cause any effect on ionization. The analysis of six independent blank human plasma samples showed no interfering peaks in the SRM traces for ibrutinib and dihydrodiol-ibrutinib and their deuterated IS.

The signals of the LLOQ level were easily distinguishable from blank, as shown in Fig. 1. A small, yet constant signal was shown in the signals of ibrutinib and dihydrodiol-ibrutinib. This seemed to originate from the internal standards, as it was absent when blank plasma extracts were injected. There was a correction for this area in the calibration curve. Blank ibrutinib and dihydrodiol-ibrutinib responses were all <20% of the LLOQ response as required [17]. Blank IS responses were below 1% (0.01% for both compounds) of the normal response. At the LLOQ of 5 ng/ml ($n = 6$) a mean concentration of 4.9 ± 0.4 ng/ml for ibrutinib, and 5.7 ± 0.9 ng/ml for dihydrodiol-ibrutinib (resp. 97.1 ± 7.3%, and 114.9 ± 16.5%) were found, justifying the investigated LLOQ level in human plasma [18]. The results of six different spiked plasma samples are shown in Table 4. For mice, in 6 different donors, the mean concentration at a LLOQ of 5 ng/ml ($n = 6$) was 5.7 ± 0.65 ng/ml for dihydrodiol-ibrutinib, and 5.8 ± 0.8 ng/ml for ibrutinib (resp. 113.8 ± 11.4 %, and 116.4 ± 13.9%). The carry-over of the signal observed on the retention time of ibrutinib and dihydrodiol-ibrutinib was below 20 % as compared to the one found at the LLOQ after the injection of two blank samples.

3.2.4. Recovery and matrix effect

The matrix effect of ibrutinib and dihydrodiol-ibrutinib in human plasma were measured as the response of QC levels versus spiked blank plasma extracts. Recovery was measured as the response of spiked blank plasma versus neat samples. Results are shown in Table 5. For mouse samples, the matrix effect was measured as relative response during continuous infusion of 5000 ng/ml at 5 μl/min (0.42 ng/s) ibrutinib, dihydrodiol-ibrutinib and their internal standards. Injections of blank mouse plasma extracts were compared to 50% v/v methanol. Ibrutinib showed a relative matrix effect of 105.6 ± 4.7%, dihydrodiol-ibrutinib 102.2 ± 3.7% during infusion. A visual representation of the data is shown in Fig. 4.

Table 4
Results ibrutinib and dihydrodiol-ibrutinib (DHI) analysis of spiked human plasma samples using plasma of six individual donors.

Level (ng/ml)	Compound	Accuracy (%)
4000	DHI	93.6 ± 4.1
	Ibrutinib	92.5 ± 3.2
400	DHI	94.9 ± 4.9
	Ibrutinib	92.7 ± 3.2
15	DHI	114.7 ± 7.7
	Ibrutinib	99.8 ± 6.8
5	DHI	114.9 ± 16.5
	Ibrutinib	97.1 ± 7.3

3.2.5. Stability

As stated in Section 3.1.4, ibrutinib, but not the dihydrodiol-metabolite, shows instability in human plasma even at room temperature (Table 1). However, when prepared, thawed and handled on ice, and stored at −30 °C, no signs of degradation could be observed. For three freeze–thaw cycles, no degradation of ibrutinib and the metabolite was found. For human plasma samples stored for three months, slight degradation could be seen (>85% remaining). Data are shown in Table 6. To assess auto-sampler stability calibration and QC samples were re-injected after additional storage at 4 °C for 24 h resulted again in successful performances without any loss of precision and accuracy. With only 1 value out of 24 exceeding 100 ± 15% (or 100 ± 20% for LLOQ), QC failures remained far below a 33% frequency (Table 7) as required by the FDA and EMA [17,18]. The responses of ibrutinib and dihydrodiol-ibrutinib from the stock solutions after 2 months at −30 °C were compared to fresh stock solutions, after appropriate dilution of the samples, using LC–MS/MS. Recoveries of ibrutinib and dihydrodiol-ibrutinib in stock solutions were 104.5% and 99.3% after 2 months

Table 5
Recovery and matrix effect of ibrutinib and dihydrodiol-ibrutinib (DHI). Recovery was measured as the response of QC levels versus spiked blank plasma. Recovery was measured as the response of spiked blank plasma versus neat samples.

Compound	Level (ng/ml)	Recovery (%)	Matrix effect (%)		
			Compound	Internal standard	Ratio
Ibrutinib	4000	99.8 ± 2.6	102.8 ± 5.7	101.6 ± 6.5	101.1 ± 2.3
	400	104.2 ± 2.7	105.1 ± 5.6	103.8 ± 5.2	101.3 ± 1.6
	15	102.8 ± 7.7	119.2 ± 8.4	110.1 ± 11.5	108.6 ± 5.0
DHI	4000	99.7 ± 2.4	103.8 ± 2.3	102.5 ± 4.2	101.3 ± 3.2
	400	102.6 ± 2	104.5 ± 6.5	104.6 ± 6.1	100.0 ± 1.6
	15	103.6 ± 11.9	114.1 ± 10.4	105.7 ± 11.0	108.1 ± 7.3

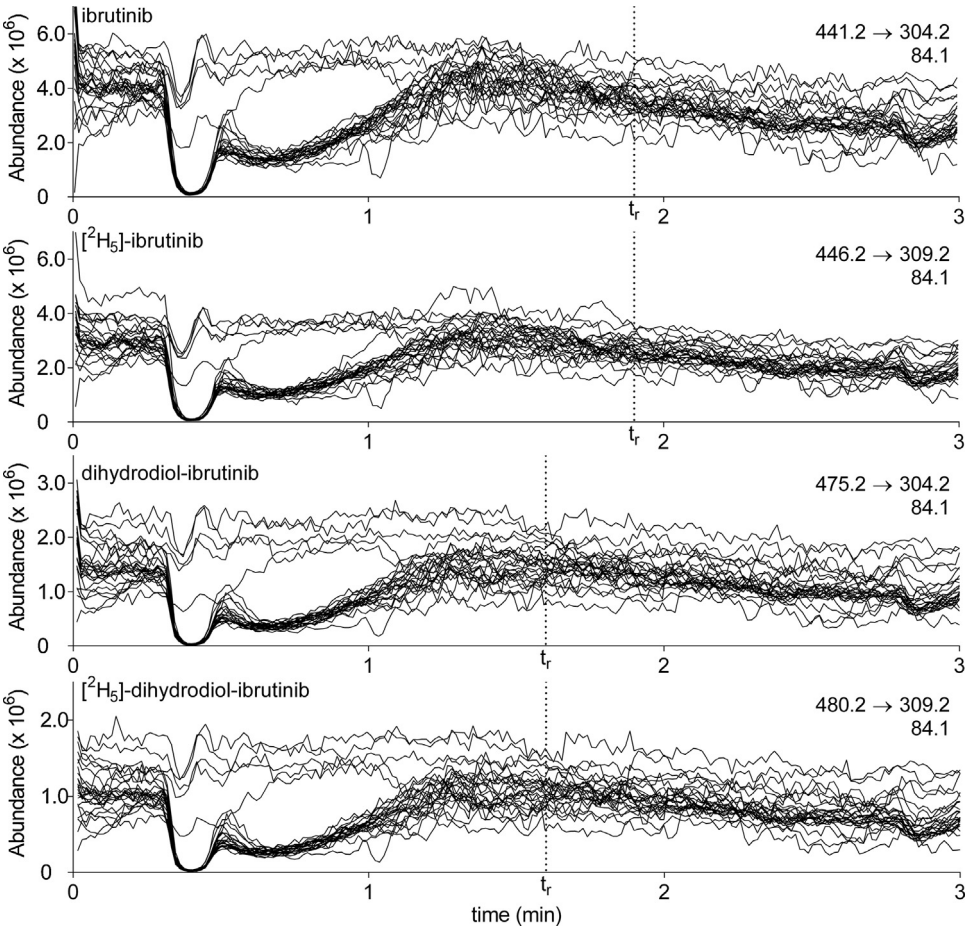

Fig. 4. MRM-Signal of ibrutinib, dihydrodiol-ibrutinib and their respective internal standards during continuous infusion. During each run blank mouse plasma extract or solvent was injected onto the column. The dashed lines represent retention times (t_r) of ibrutinib (1.9 min) and dihydrodiol-ibrutinib (1.6 min).

Table 6
Stability (mean ± SD) of QC low (15 ng/ml) and high (4000 ng/ml) samples after various storage conditions.

Storage condition	Species	DHI (%)		Ibrutinib (%)	
		Low (15)	High (4000)	Low (15)	High (4000)
3 Freeze–thaw cycles	Human	101.8 ± 3.7	107.9 ± 5.6	105.6 ± 3.2	97.9 ± 5.7
	Mouse	91.6 ± 7.4	100.2 ± 3.8	99.5 ± 13.8	101.9 ± 1.6
3 Months (-30 C)	Human	104.9 ± 6.3	86.8 ± 1.8	86.5 ± 0.3	95.7 ± 6.2

at −30 °C (n = 2), respectively. Results of both experiments were therefore considered satisfactory (100 ± 5%) for the validation.

3.3. Pharmacokinetic study in mice

After validation of this method, the assay was used to perform a pilot pharmacokinetic study of ibrutinib and dihydrodiol-ibrutinib in five female wild-type FVB mice. Herein we demonstrated that an oral dose of 10 mg/kg ibrutinib is rapidly absorbed (t_{max}: 7.0 ± 4.5 min, C_{max}: 608.8 ± 275.6 ng/ml, k_a 2.3 ± 0.8 min, AUC_{0-inf}: 25.2 ± 6.5 μg/ml min), and is rapidly eliminated ($t_{½}$: 36.5 ± 6.1 min). Elimination is mainly in the form of the active metabolite dihydrodiol-ibrutinib ($t_{½}$: 64.9 ± 6 min, t_{max} 27.0 ± 19.6 min, C_{max}: 1194.5 ± 351.0 ng/ml, AUC_{0-inf}:

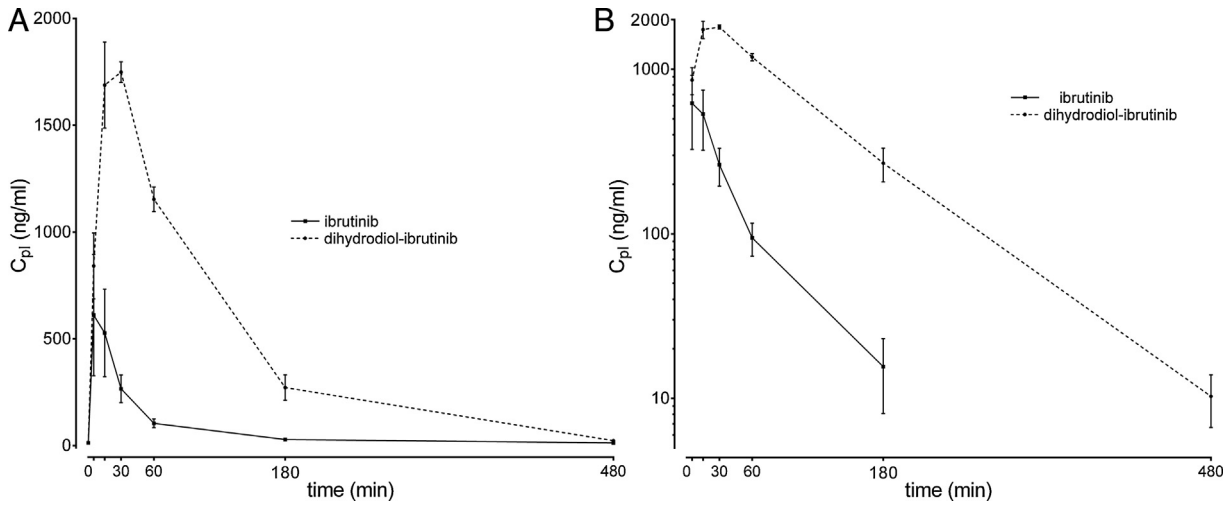

Fig. 5. Ibrutinib plasma concentration–time curve of pilot pharmacokinetic study in FVB mice.

Table 7
Auto sampler stability, difference (mean ± SD) of QC's after 24 h storage ($n = 6$ per level).

QC	Compound	Difference (%)
High (4000 ng/ml)	Ibrutinib	0.2 ± 2.3
	DHI	1.4 ± 2.2
Medium (400 ng/ml)	Ibrutinib	0.9 ± 3.5
	DHI	1.4 ± 3.1
Low (15 ng/ml)	Ibrutinib	−4.7 ± 4.6
	DHI	7.5 ± 5.6
(5 LLOQ ng/ml)	Ibrutinib	−8.6 ± 7.1
	DHI	3.6 ± 8.6

152.7 ± 53.9 μg/ml min), which has an inhibitory effect on BTK, but does not bind irreversibly due to the loss of the highly reactive acrylamide group [4,13]. Data are represented as average pharmacokinetic parameter ± SD. The plasma concentration–time curves are shown in Fig. 5. The pharmacokinetic study presented in this study is a pilot study and the pharmacokinetic parameters presented cannot be accepted as true PK parameters for ibrutinib and its metabolite in FVB mice. The results from the pilot study will be used to design the final PK study and a final PK study in FVB mice will be conducted.

4. Conclusion

A LC-ESI-MS/MS method has been developed for the rapid and precise quantitation of ibrutinib and dihydrodiol-ibrutinib in human and murine plasma samples from pharmacokinetic studies. The method was successfully validated to meet our needs, as demonstrated in the pilot pharmacokinetic study. Results show that accuracy, precision and stability were within the limits as described within international guidelines [17,18]. In the pharmacokinetic analysis, no sample was found higher than the ULOQ, and only samples lower than the LLOQ were found after 480 min, indicating that the chosen concentration range is adequate. Ibrutinib, not dihydrodiol-ibrutinib, has shown to be unstable in human plasma at 30 °C, or even room temperature. Ibrutinib is most likely broken down by loss of the acryl moiety on the piperidine ring, resulting in a molecule with a m/z of 401.2. However, following three freeze–thaw cycles and prolonged sample storage, samples can be safely stored for 3 months at −30 °C, provided that they are handled on ice.

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