Bioanalysis of ibrutinib, and its dihydrodiol- and glutathione cycle metabolites by liquid chromatography-tandem mass spectrometry

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ABSTRACT

Ibrutinib is a targeted covalent inhibitor frequently used for the treatment of various lymphomas. In addition to oxidative metabolism, it is metabolized through glutathione coupling. The quantitative insight into this kind of metabolism is scarce, and tools for quantitation are lacking. The non-oxidative metabolism could prove a more prominent role when oxidative metabolism is impaired. Also, *in-vitro* studies could over-estimate the effect of CYP450-inhibition. To gain quantitative insight into this relatively unknown biotransformation pathway of the drug we have developed a validated simple, fast and sensitive bio-analytical assay for ibrutinib, dihydrodiol-ibrutinib, and the glutathione, cysteinylglycine and cysteine conjugates of ibrutinib in human plasma. The method emphasizes on simplicity, the thiol-conjugates were synthesized by a simple one step synthesis, followed by LC-purification. Sample preparation was done by a simple protein crash with acetonitrile containing labeled internal standards, evaporation of solvents, and reconstitution in eluent. Finally, the compounds were quantified using UHPLC–MS/MS. The assay was successfully validated in a 0.5–500 nM calibration range for all compounds, and also a lower range of 0.05–50 nM was demonstrated for ibrutinib to accommodate for even the lowest trough levels. This assay has a considerably higher sensitivity than previous published assays, with the previous lowest LLOQ being 1.14 nM. Both, ibrutinib, dihydrodiol-ibrutinib and the cysteine conjugate were deemed stable under refrigerated or frozen storage conditions. At room temperature, the glutathione conjugate showed rapid degradation into the cysteinylglycine conjugate in plasma. Finally, the applicability of the assay was demonstrated in patient samples.

1. Introduction

Recently, much progress has been made into the development of targeted covalent inhibitors. These inhibitors interact with their target through non-covalent interaction of the pharmacophore, after which the Michael acceptor, often α,β-unsaturated acrylamides, bind covalently to a free cysteine residue in the binding pocket of the targeted protein [1]. Ibrutinib has an exceptional inhibiting potency for the target, Bruton's tyrosine kinase (BTK). The drug has been designed to form a covalent bond with cysteine-481 when presented in a favorable position in the active site of BTK [2]. It is a first in class BTK inhibitor which inactivates the NFκB pathway, and is registered for mantle cell lymphoma, chronic lymphocytic leukemia, Waldenström's macroglobulinemia [3], marginal zone lymphoma, and chronic graft *versus* host disease [4].

While Cytochrome P450 mediated oxidative metabolism of

ibrutinib to dihydrodiol-ibrutinib (DHI) is well known [5], the knowledge on pharmacokinetics (PK) through (extrahepatic-) clearance by either direct or enzymatic conjugation through glutathione S-transferase (GST) of ibrutinib to glutathione (GSH) is limited. The GSH tripeptide consists of glutamate (GLU), cysteine (CYS) and glycine (GLY), and is present in millimolar concentrations in most cells. Through γ-glutamyl transferase (γ-GT) GSH-conjugates are rapidly metabolized to cysteinylglycine (CGS)-conjugates which are further converted into CYS-conjugates by dipeptidases [6]. The quantitative insight into this kind of metabolism is scarce, and tools for quantitation are lacking. Preclinical studies utilizing cellular or microsomal systems to evaluate CYP450 mediated metabolism of compounds like ibrutinib may overestimate the contribution of these metabolites to the clearance of ibrutinib *in-vivo*. Because of this over-estimation, non-oxidative metabolism through glutathione could have a more prominent role *in-vivo* than was previously thought, especially when oxidative metabolism is impaired [7]. The *in-vitro* formation of ibrutinib-GSH (IGSH) conjugates has been previously described [8]. An imbalance between predicted clearance from hepatocyte incubations and total body clearance of ibrutinib was observed. This was attributed to extrahepatic conjugation to GSH, which was not captured in the hepatocyte *in-vitro* system. However, IGSH is highly unstable in biological samples and no analysis of downstream thio-metabolites (ISMTs) was performed, leading to an underestimation of this metabolic pathway. IGSH is most likely rapidly converted to ibrutinib-CGS (ICGS) due to γ-GT activity in plasma or drug metabolizing organs. The inclusion of ICGS and also ibrutinib-CYS (ICYS) metabolites thus provides a more detailed view of this pathway.

Due to the reactive nature of the acrylamide group, ibrutinib-thiol conjugate metabolites can easily be synthesized. Ibrutinib reacts instantly to free thiol groups in a favorable environment (*e.g.* at a pH above the pK_a (*ca.* 9) of the thiol). These reactions occur at mild, ambient conditions, and in the presence of water. The simplicity of these reactions, along with a complete reaction of ibrutinib and minimal by-products allows for minimal purification afterwards. In this paper we present a novel, rapid and precise method to synthesize and quantify the three main metabolites of the GSH-mediated biotransformation pathway of ibrutinib. A number of assays for the bioanalysis of ibrutinib and DHI have been developed in recent years [9], but not for IGSH, ICGS and ICYS. Further, the method we present is able to quantify lower quantities of ibrutinib and DHI than any of the previously published methods [9–14]. The previous lowest quantification limits are 1.14 nM for ibrutinib and 1.05 nM for DHI (both 0.5 ng/mL) by LC-MS/MS analysis [10]. An assay using ultra-high-performance liquid chromatography (UHPLC)-diode array detection had a quantitation limit of 11.4 nM, which is unsuitable for analysis of plasma trough-levels [13]. The new assay will be used to gain more insight into the extrahepatic metabolism of ibrutinib in a future study.

2. Materials and methods

2.1. Chemicals and reagents

Ibrutinib (> 99.9%, M_w: 440.51 g/mol) was acquired from LC Labs (Wyoming, MA, USA). The main metabolite DHI (95.9%, M_w: 474.52 g/mol) and the respective [$^2H_5$]-labeled ibrutinib (acetate salt, > 99.9%, M_w: 445.54 g/mol), and dihydrodiol-ibrutinib (> 99.9%, M_w: 479.53 g/mol) were obtained from Alsachim (Illkirch Graffenstaden, France). Methanol of HPLC quality and acetonitrile of HPLC-S gradient grade quality were from Biosolve (Valkenswaard, The Netherlands). Water for all purposes was purified in-house by a Synergy UV apparatus (Merck Millipore, Darmstadt, Germany). Analytical grade dimethyl sulfoxide (DMSO) was supplied by VWR (Fontenais-sous-Bois, France). Cysteinylglycine (≥95%) was purchased from Bachem (Bubendorf, Switzerland). Formic acid (≥98%), cysteine-HCl (≥98%), reduced glutathione (≥98%), and all other chemicals originated from Sigma-Aldrich (Steinheim, Germany). Human lithium-heparinized plasma was

obtained from Sera Laboratories (Haywards Heath, West Sussex, UK). For the evaluation of selectivity and matrix effect, human lithium-heparinized plasma of six individual donors was obtained from Innovative Research (Novi, MI, USA).

2.2. Synthesis of small molecular thiol-conjugates

Approximately 1 mg ibrutinib was dissolved in 2 mL methanol (2.27 mM) and a 113.5 mM stock solution of CYS, CGS, or GSH was added at a 1:25 molar ratio. Finally, the mixture was diluted to 567.5 μM (0.25 mg/mL) with water, and the pH was adjusted to 10 using concentrated NH_4OH. The mixture was incubated at room-temperature for 12 h. The formation of ICYS, ICGS, and IGSH, as well as the residual concentration of free ibrutinib were followed by monitoring the $[M + H]^+$ ions at *m/z* 441.2, 562.1, 619.1, and 749.3 by UHPLC/MS. At the end of the reaction, the mixture was subjected to purification on a ReproSil-pur 120 C18-AQ column (250 × 10 mm, 10 μm particle size, Dr. Maisch, Ammerbusch, Germany) using a Shimadzu LC10 LC system with a SPD10Av UV/VIS detector (Shimadzu, 's-Hertogenbosch, The Netherlands) equipped with a Gilson 215 fraction collector (Gilson, Middleton, WI). Mobile phase A was 0.1% (v/v) formic acid in water, and mobile phase B 0.1% (v/v) formic acid in acetonitrile, with a flow rate of 6 mL/min. The mobile phase gradient, described by solvent B percentage, was held at 20% B for 4 min, and was then increased linearly to 50% B at 50 min, and further to 100% at 51 min. From 52 to 53 min it was reduced linearly to 20% resulting in a total run time of 60 min after 7 min of equilibration. The fractions were collected based on their UV absorption (214 and 256 nm) and were combined after confirmation with mass spectrometric data ($[M + H]^+$ at *m/z* 562.1, 576.1, and 748.2). The ibrutinib-conjugates were pre-concentrated by rotary evaporation (Buchi R100, Flawil, Switzerland). After dilution in *tert*-butanol/water (1:1, v:v), the compounds were freeze-dried using a Christ Alpha 2–4 LSC lyophilizator (Osterode am Harz, Germany). The synthesized compounds were stored in sealed vials under nitrogen with a desiccant at −30 °C. The purity of each of the compounds was evaluated by UHPLC-UV at 214 nm.

2.3. Chromatography and MS/MS method

To quantify the formation of ibrutinib conjugates, standards were synthesized (see Section 2.2). The chromatographic separation of the UHPLC-MS/MS method was performed on a binary UHPLC system, consisting of two LC-30AD pumps, a SIL30-ACmp auto-sampler, a CTO-20AC column oven, and a DGU-20A5R degasser, all from Shimadzu. The method consisted of a linear gradient of 0.1% (v/v) formic acid in water, and methanol, with a run-time of 10 min. The solvent composition was held at 20% organic modifier for 0.5 min, after which it was increased linearly to 40% at 0.6 min, to 57% at 4.0 min, to 80% at 7.0 min, and to 95% at 7.5 min. At 8.0 min, the composition was reverted back to 20%, with a total run time of 10 min. Separation was performed on a Waters Acquity CSH-C18 column (2.1 × 100 mm, 1.7 μm particle size, Waters, Milford, USA). Calibration samples were prepared in human blank plasma from a combined DMSO stock solutions of 50 μM ibrutinib, DHI and the ISMTs, and ranged from 0.5–500 nM. Samples exceeding this range were diluted accordingly using blank plasma. For detection a Sciex QTRAP® 5500 triple quadrupole mass spectrometer, with Analyst 1.6.2, and MultiQuant 3.0.1 software (Sciex, Ontario, Canada) was used. Settings used for the ionization source were curtain gas, 40 psi; collision gas, "medium"; ion-spray voltage, 5000 V; temperature, 300 °C; ion source gas 1, 90 psi; ion source gas 2, 90 psi. Dwell times were 5 msec, and entrance potential was set to 10 V; compound specific parameters can be found in Table 1. The Q1 *m/z* for ibrutinib was shifted to the +1 isotope (*m/z* 442.2) to avoid saturation of the continuous electron multiplier, and thus improve linearity. Stable isotope ($^2H_5$) labeled DHI was used as an internal standard for DHI and the ISMTs. For ibrutinib, $^2H_5$-labeled ibrutinib

Table 1
Tuned MS/MS parameters for all quantified components. The entrance potential was set at 10 v for all compounds, dwell-time was 5 ms.

Compound	*m/z* Q1	*m/z* Q3	DP (V)	CE (V)	CXP (V)
Ibrutinib	442.2	304.1	211	45	12
Ibrutinib (low range)	441.2	304.1	211	45	12
		138.1	211	35	16
DHI	475.2	304.1	221	45	12
		84.1	221	73	10
[^{2}H$_5$]-ibrutinib	446.2	138.1	196	35	16
		84.1	196	71	10
[^{2}H$_5$]-DHI	480.2	309.1	86	45	14
		84.1	86	77	10
ICYS	562.2	473.1	126	39	20
		304.1	126	57	14
ICGS	619.1	473.0	216	41	20
		304.1	216	63	14
IGSH	749.3	620.2	106	31	26
		474.2	106	51	18

DP: declustering potential, CE: collision energy, CXP: collision cell exit potential.

was used.

2.3.1. Data processing

For chromatographic data, Analyst 1.6.2, and MultiQuant 3.0.1 software (Sciex) were used. For further data-processing Microsoft Excel 2016 for Mac (version 15.25), the statistical software R, version 3.3.2 [15], RStudio, version 1.0.136 [16], and GraphPad Prism 6 for Mac OS X, (version 6.0h) were used.

2.4. Standard solutions and quality controls

For each compound, a stock solution of 1 mM was prepared by weighing 300 to 500 μg ibrutinib, DHI, IGSH, ICGS or ICYS, and dissolving this in an appropriate volume of DMSO. Sequential dilution to a 50 μM DMSO working solution containing all compounds was used for calibration samples in human plasma. Before each use the calibration samples were prepared in blank human plasma on wet ice by dilution of the 50 μM working solution to produce concentrations of 500, 250, 100, 50, 10, 5, 1, and 0.5 nM of each compound.

Quality control (QC) samples were prepared from a separate 50 μM working solution in DMSO, which was diluted using blank plasma to obtain QCs at 400 (high), 100 (medium), 2 (low) and 0.5 (lower limit of quantitation, LLOQ) nM in blank human plasma. QCs were prepared freshly before use, on wet ice, in a similar fashion to calibration samples.

2.5. Sample preparation

For sample preparation, 200 μL of acetonitrile containing 1.5 nM ^{2}H$_5$-ibrutinib and ^{2}H$_5$-DHI, and 0.1% (v/v) formic acid was added to a 100-μL sample in a 1-mL well of a 96-deep well plate. The plate was vortex-mixed at 1000 rpm for 5 min, after which the plates were centrifuged for 5 min at 3500 RCF. Two-hundred μL of the supernatant was

transferred to a 96-well microtiter plate. The solvent was evaporated to approximately 25 μL under a gentle nitrogen stream (Porvair MiniVap, 40 L/min, 40 °C). The samples were diluted with 100 μL water/methanol/formic acid, (80:20:0.08, v/v/v), and vortex-mixed for 5 min at 1000 rpm.

2.6. Analytical method validation

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

2.6.1. Calibration

Combined calibration solutions of ibrutinib, DHI, IGSH, ICGS, and ICYS were prepared by diluting a working solution containing all compounds at 50 μM to 500 nM in human lithium heparin plasma. These solutions were diluted directly in 1.5 mL polypropylene tubes. The calibration samples were prepared from these solutions as described in Section 2.4, and were processed in duplicate for each daily calibration. Least squares linear regression was used to define the calibration curves, using the analyte/IS peak ratios.
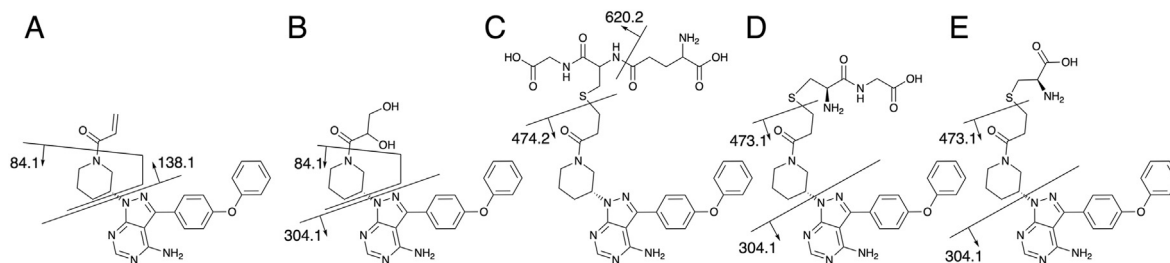
2.6.2. Precision and accuracy

A 50 μM working solution of ibrutinib, DHI, IGSH, ICGS, and ICYS in DMSO was used to produce the QC and LLOQ samples in human plasma (400, 100, 2, and 0.5 nM). Precisions and accuracies 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. A single accuracy and precision run on one day was performed for a lower range at the QC-levels of 50, 10, 2, 0.5, 0.15, and 0.05 nM ibrutinib. Standards of 0.25, 0.1, 0.05 nM ibrutinib were added to the calibrations, and 500 and 100 nM were not used. For sample pretreatment, acetonitrile containing 0.15 nM ^{2}H$_5$-ibrutinib and 0.1% (v/v) formic acid was used in this case.

2.6.3. Selectivity

To assess the selectivity, six individual human plasma samples were processed to test the selectivity of the assay. Samples were processed without analytes and IS and with all analytes at the LLOQ level (0.5 nM), supplemented with the IS.

2.6.4. Stability

The stability of ibrutinib, DHI, IGSH, ICGS, and ICYS was investigated in QC-high (400 nM), −medium (100 nM) and -low (2 nM) plasma samples stored in polypropylene tubes. Quadruplicate analysis of human samples was performed after storage at 0 °C for 2 h. Separate samples were subjected to three freeze-thaw cycles (thawing at 0 °C, during *ca.* 2 h and freezing again at −80 °C for at least 12 h), and then analyzed. Further, a set of separate tubes was analyzed after 2 months at −80 °C to evaluate long term stability. To test the stability at the conditions in the auto injector, an analytical run was re-injected after additional storage of the extracts at 4 °C for 48 h. Finally, the responses of ibrutinib, DHI, IGSH, ICGS, and ICYS from the stock solutions in DMSO after 1 month at −80 °C (n = 2) were compared to fresh stock

Fig. 1. Proposed fragmentation patterns for A) ibrutinib, B) DHI, C) IGSH, D) ICGS, E) ICYS.
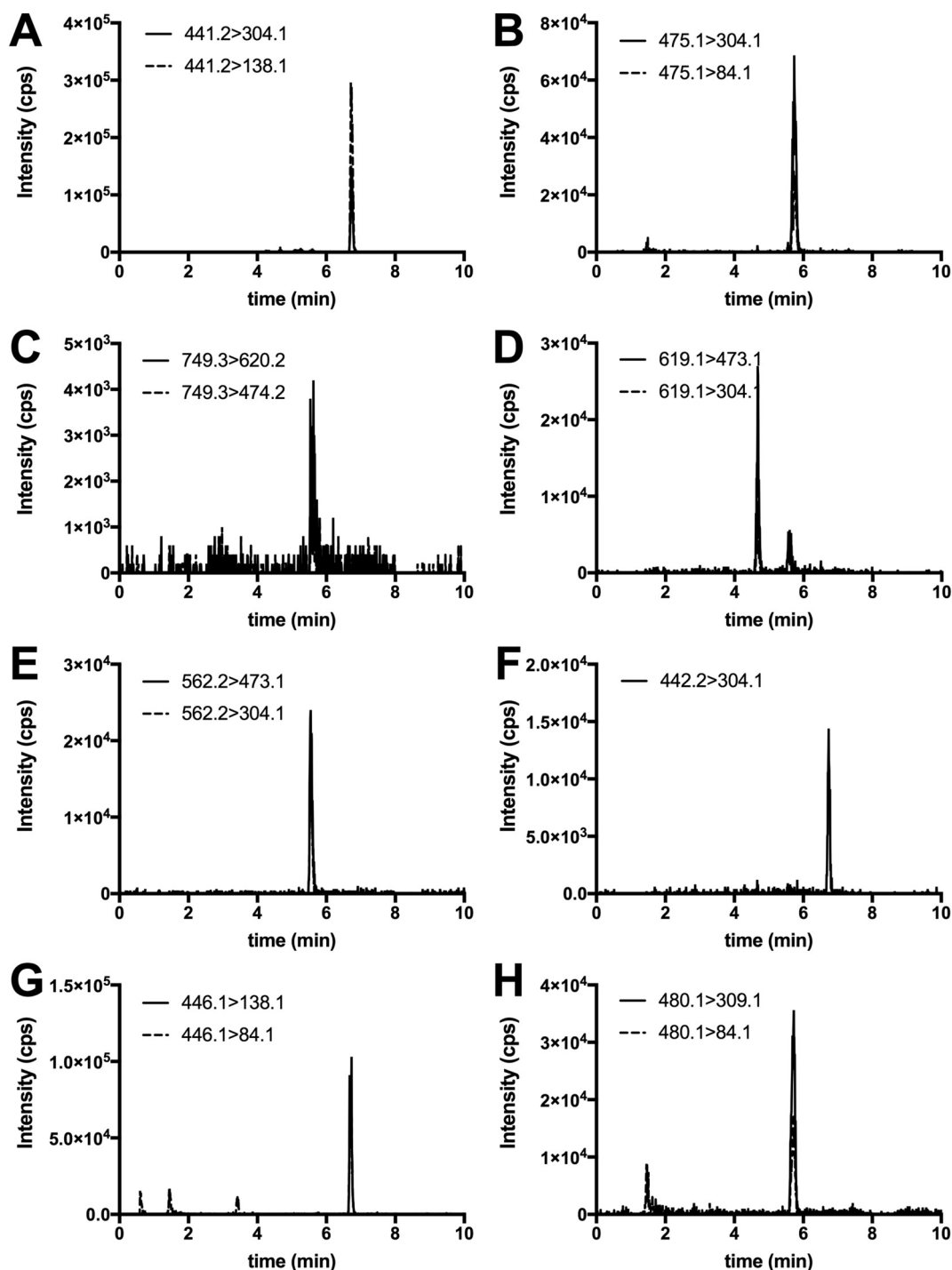
Fig. 2. Chromatograms of ibrutinib (A), DHI (B), IGSH (C), ICGS (D), ICYS (E), Ibrutinib at precursor m/z $[M + H]^+$ $+ 1$ (F), $[^2H_5]$-ibrutinib (G), $[^2H_5]$-DHI (H) in human plasma at 0.5 nM, internal standards at 1.5 nM.

solutions with UHPLC-MS/MS after appropriate dilution of the samples with 50% (v/v) methanol and adding internal standards.

2.7. Analysis of ibrutinib, DHI and ISMT-conjugate in human plasma

As part of a TDM program, five plasma samples of a 75-year-old male patient receiving 420 mg ibrutinib once daily were analyzed for ibrutinib, DHI, IGSH, ICGS, and ICYS. Samples were prepared as described above. After drawing blood, the samples were kept on ice, plasma and cells were separated at 4 °C within 1 h, and lithium-heparin plasma samples were kept on ice to prevent degradation of ibrutinib and the ISMT-conjugates. The samples were stored at −80 °C until analysis. For the incurred sample reanalysis, a set of 24 plasma samples from two patients were analyzed in duplicate. These samples were reanalyzed three days later. The incurred sample reanalysis was calculated as (repeat value − initial value) / mean value × 100%. The repeated analysis should not differ > 20% of the mean for at least 67% of the repeats [17].

3. Results and discussion

3.1. Synthesis of small molecular thiol-conjugates

For all reactions, < 1% free ibrutinib remained after the incubation. The SMT-conjugates had a purity of at least 97%, and a yield above 70% after purification on a small scale preparative LC, and subsequent removal of solvent through lyophilization.

3.2. Method development

3.2.1. Chromatography and MS/MS method

Positive ESI-MS/MS settings were optimized for the protonated compounds and their deuterated internal standards. For ibrutinib the precursor m/z value was raised by 1, the product m/z value was left unchanged. The normal $[M + H]^+$ signal intensity for ibrutinib (the compound with the highest sensitivity) was a lot higher than that of IGSH (the compound with the lowest concentrations and also sensitivity). This posed a problem, as we increased the signal intensity to reach the LLOQ of 0.5 nM for IGSH, we caused signal saturation for ibrutinib somewhere between the concentrations 100 to 250 nM. To alleviate this problem, we shifted Q1 m/z of ibrutinib to the ^{13}C-isotope, which is 27% of the ^{12}C-isotope intensity. For the other compounds, the sums of the two most prominent dissociation products for each compound were measured for maximal sensitivity (except for ibrutinib, where only one mass transition, m/z 442.2 > 304.1, was used for quantitation). The mass transitions are shown in Table 1. Product spectra of all compounds are shown in Fig. 1. 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 chromatographic results. Retention times of ibrutinib and DHI were 6.72 and 5.73 min. For IGSH the retention time was 5.64 min, for ICGS 4.68 min, and for ICYS 5.55 min. Representative chromatograms of extracted human plasma are shown in Fig. 2. Retention-times of the internal standards closely matched those of the native compounds. Based on retention time stability (RSD < 1% for both compounds), the column was sufficiently equilibrated.

3.2.2. Sample preparation

The developed method used as few steps as possible, eliminating labor intensive steps and reducing errors. A simple protein precipitation was chosen over more labor-intensive liquid-liquid or solid-phase extraction methods. Salt assisted liquid-liquid extraction can be a more selective alternative for kinase inhibitors, but for this the extracts would need to either be diluted with water or all solvent has to be completely evaporated and reconstituted in eluent for compatibility with the chromatographic system [19–21]. Dilution would lower the concentration in the final extracts. Evaporating the solvent and reconstituting in eluent could pose more troublesome, since reconstitution in the initial mobile phase could be incomplete. The extracts would primarily consist of acetonitrile, and will rapidly be evaporated to complete dryness, while for the current method most of the acetonitrile was evaporated, but some residual solvent remained. This aided in improvement of the extraction recovery. The protein precipitation with a 1:2 (v/v) ratio of sample:acetonitrile gave a good recovery and extraction of the compounds, while matrix effects were small and/or circumvented by the use of internal standards, or could be neglected because of the reproducible and identical matrix effect of the calibration and QC samples (see Section 3.3.5 for more detail).

3.3. Analytical method validation

In our assay, an upper limit of quantitation (ULOQ) was proposed at 500 nM. This level was chosen after the LLOQ was determined at 0.5 nM for the ISMT metabolites, and a range of three orders of magnitude could be analyzed in a linear fashion. Because of the higher

Table 2
Accuracies and precisions of ibrutinib, DHI, IGSH, ICGS, and ICYS in human plasma at high range (0.5–500 nM, 3 days, mean $\pm$ sd, n = 6 per level).

	Level (nM)	Accuracy (%)	Within day (%)	Between Day (%)
Ibrutinib	400	102.0	6.1	7.5
	100	103.8	5.2	8.7
	2	103.8	9.7	12.3
	0.5	100.9	10.1	10.7
DHI	400	99.7	3.0	4.9
	100	104.2	5.4	5.4
	2	104.8	4.5	7.6
	0.5	101.6	7.1	10.5
ICYS	400	109.8	3.8	5.4
	100	103.6	6.4	7.3
	2	104.9	11.0	11.5
	0.5	102.5	9.5	9.8
ICGS	400	97.1	4.4	6.0
	100	107.1	4.3	4.3
	2	109.8	7.4	8.4
	0.5	102.5	11.0	11.4
IGSH	400	100.9	4.1	7.1
	100	105.1	3.6	4.5
	2	105.4	6.2	6.9
	0.5	100.4	6.9	7.3

response for ibrutinib without the mass shift, it could also be analyzed at a 10-fold lower concentration of 0.05–50 nM (0.022–22 ng/mL) to accommodate even the lowest trough-levels in therapeutic drug monitoring, for this range 0.25, 0.10 and 0.05 nM calibration samples were added, and the internal standard concentration ([^{2}H$_5$]-ibrutinib) was lowered to 0.15 nM. The lower range was only evaluated for accuracy and precision, as it will not be routinely used. This range was substantially lower than the previous methods with the lowest LLOQ being 1.14 nM for ibrutinib [10].

3.3.1. Calibration

The calibration showed linear results for all compounds, with R^2's over 0.97 deemed as acceptable. Most curves show an R over 0.99 with linear $1/X^2$ weighted regression, showing the validity of the chosen range and the m/z shift of ibrutinib, as using the 441.2 > 304.1 transition would reach saturation of the signal between 100 and 250 nM.

3.3.2. Precision and accuracy

In Tables 2 and 3 the accuracies and precisions of the method are shown. The precisions and accuracies for three analytical runs were within the $\pm$ 15% for high, medium, and low QCs, and $\pm$ 20% for the LLOQ QCs, as required [13,14].
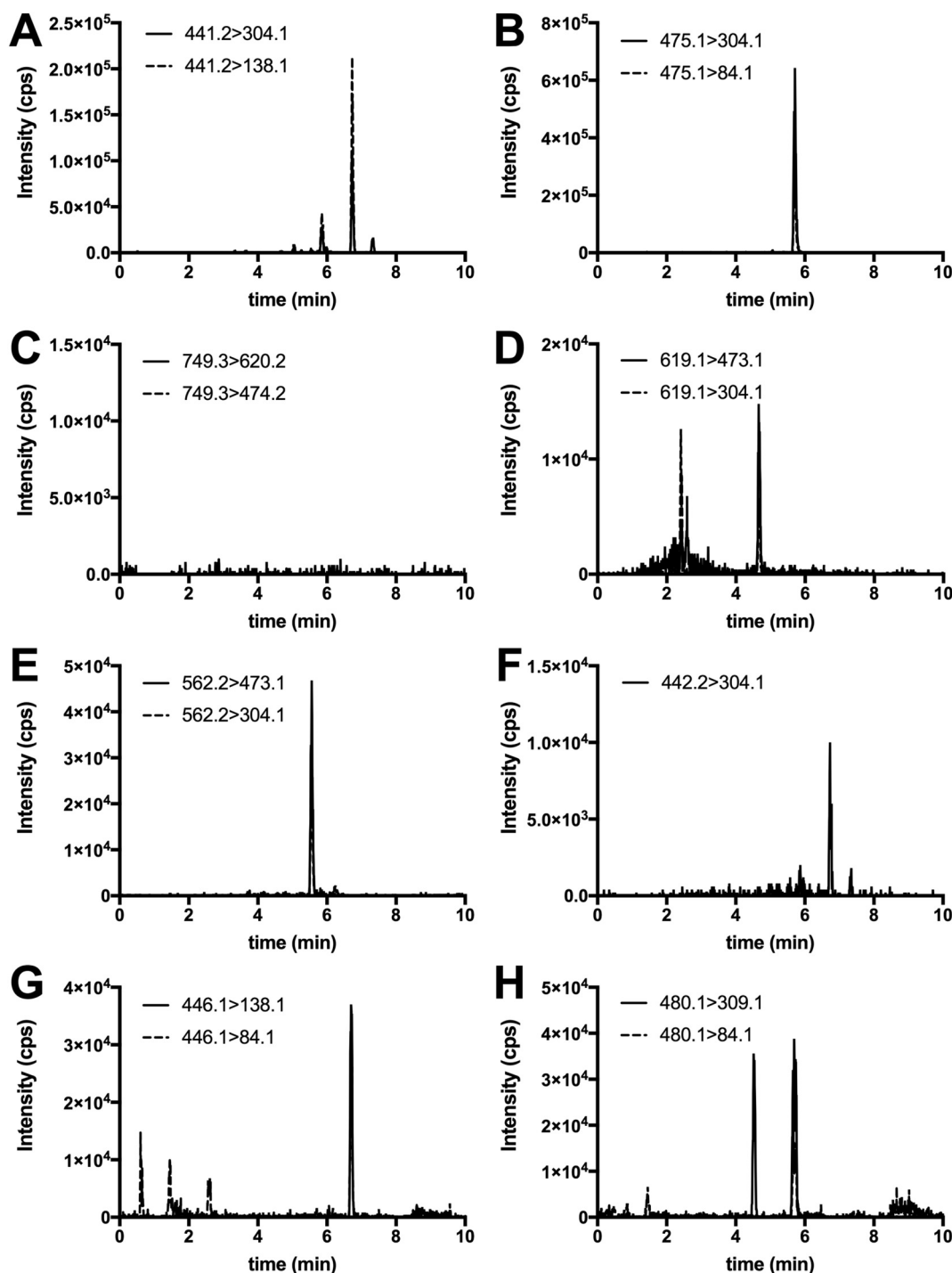
3.3.3. Selectivity

All blank samples gave a response lower than 20% of the LLOQ, showing good selectivity. At the LLOQ of 0.5 nM, mean concentrations were found to be 0.55 $\pm$ 0.07 nM for ibrutinib, 0.56 $\pm$ 0.04 nM for DHI, 0.50 $\pm$ 0.06 nM for IGSH, 0.48 $\pm$ 0.07 nM for ICGS, and 0.48 $\pm$ 0.06 nM for ICYS (109.5 $\pm$ 12.1, 112.5 $\pm$ 8, 100.4 $\pm$ 12.5, 96.5 $\pm$ 14.2 and 95.8 $\pm$ 12.9% respectively). For ibrutinib and DHI,

Table 3
Accuracies and precisions of ibrutinib, in human plasma at low range (0.05–50 nM, mean $\pm$ sd, n = 6 per level).

Level (nM)	Precision (%)	Accuracy (%)
50	3	104
10	0	100
2	3	99
0.5	1	103
0.15	12	101
0.05	12	111

Fig. 3. Patient-sample chromatograms of ibrutinib (A, 15.8 nM), DHI (B, 59.3 nM), IGSH (C, not detected), ICGS (D, 5.1 nM), ICYS (E, 12.1 nM), Ibrutinib at precursor m/z [M + H]$^+$ + 1 (F), [^{2}H$_5$]-ibrutinib (G), [^{2}H$_5$]-DHI (H) at rough-level from a patient that received 420 mg ibrutinib once daily. For [^{2}H$_5$]-DHI (H) an isobaric interference is present at 4.53 min (t$_r$ = 5.72 min).

small but constant signals (10 ± 8 and 16 ± 5% of LLOQ) were present. These originated from the non-labeled impurities in both internal standards, as addressed previously [11]. The levels of IS (1.5 nM) were chosen to limit the effect of this impurity. For the lower range, the ibrutinib signal showed similar results by lowering the IS to 0.15 nM. Blank IS responses were below 1% of the normal response for all compounds. For ^{2}H$_5$-DHI an isobaric response was found at the 480.1 > 309.1 transition at 4.7 min. This potential interference was previously identified by de Vries et al. as taurocholic acid and is mainly present in hepatically impaired patients [10]. ^{2}H$_5$-DHI and taurocholic

acid differed in retention-time so there was no actual interference (Fig. 3H).

3.3.4. Stability

While Ibrutinib, DHI, and ICYS were stable at all tested conditions by showing 87–100% recoveries, IGSH and ICGS were not. IGSH and ICGS recoveries showed little to no change for the long-term stability (96–110%), but a 50 to 70% decrease for IGSH recovery under freeze-thaw conditions was observed, while ICGS recovery shows an increase of 40 to 70%, as IGSH is most likely being converted to ICGS by the

Table 4
Relative and absolute extraction recovery for all compounds (mean ± sd, n = 4).

Extraction ratio		Ibrutinib	DHI	IGSH	ICGS	ICYS
Relative	H	96 ± 2%	94 ± 3%	81 ± 5%	96 ± 5%	92 ± 5%
	M	101 ± 5%	99 ± 4%	90 ± 3%	100 ± 4%	100 ± 4%
	L	95 ± 4%	98 ± 4%	89 ± 1%	100 ± 2%	99 ± 2%
Absolute	H	111 ± 6%	97 ± 3%	83 ± 2%	99 ± 2%	94 ± 2%
	M	90 ± 11%	95 ± 5%	88 ± 2%	97 ± 7%	97 ± 7%
	L	95 ± 5%	99 ± 8%	90 ± 6%	101 ± 6%	100 ± 6%

Table 5
Relative and absolute calculated matrix effects for all compounds (mean ± sd, n = 4).

Extraction ratio		Ibrutinib	DHI	IGSH	ICGS	ICYS
Relative	H	103 ± 5%	109 ± 6%	132 ± 3%	109 ± 3%	122 ± 3%
	M	95 ± 2%	111 ± 2%	151 ± 7%	103 ± 4%	127 ± 4%
	L	92 ± 12%	103 ± 1%	150 ± 3%	105 ± 2%	130 ± 2%
Absolute	H	88 ± 1%	118 ± 6%	143 ± 3%	119 ± 3%	133 ± 3%
	M	71 ± 3%	135 ± 5%	183 ± 4%	124 ± 3%	154 ± 3%
	L	86 ± 9%	120 ± 5%	176 ± 7%	123 ± 6%	152 ± 6%

plasma γ-GT. Samples containing IGSH were proven highly unstable under ambient and freeze-thaw conditions, with short term stability even at 0 °C being no longer than a few hours. The pretreated samples were stable at 4 °C for at least 48 h for all compounds. A run of 18 QC samples showed similar results as the initial measurement, no QCs exceeded ± 15% (or ± 20% for the LLOQ samples).

3.3.5. Recovery and matrix effect

All analytes showed a moderate to good recovery with low variation as can be seen in Table 4. For the ISMT compounds, a constant matrix effect was observed (Table 5). The use of stable isotope labeled DHI as an internal standard could not correct for this, nonetheless the calibration and QCs for these samples, as well as the selectivity experiment showed no negative effects. This was shown by the accuracy, precision and selectivity experiments.

3.4. Analysis of human plasma samples

The plasma levels of ibrutinib, DHI, and the ISMTs were measured in five samples from a patient refractory to chemotherapy, treated with a daily dose of 420 mg ibrutinib. At 22.6 ± 0.4 h post-dose, ibrutinib was found at 8.30 ± 4.05 nM in plasma. For the other metabolites, the values were: DHI: 62.48 ± 23.26, ICGS: 3.60 ± 1.77, and ICYS: 6.16 ± 3.21 nM. IGSH was not detected in plasma at trough times. Incurred sample reanalysis of 24 additional samples which are part of a separate study analyzed in duplicate showed that the method can reliably report the subject sample analyte concentrations for at least 67% of the samples, as required [17]. 83% of the ibrutinib samples showed a difference < 20%, for DHI 100%, for ICGS 83% and for ICYS 96%. For IGSH the ISR could not reliably be determined, because too many levels were around or below LLOQ.

4. Conclusions

A method to sensitively quantify ibrutinib, DHI, IGSH, ICGS, and ICYS by UHPLC-ESI-MS/MS has successfully been developed and validated. To our knowledge it is the first published validated method for this type of drug-metabolites in one assay. The method was successfully validated, as was shown by the analysis of ibrutinib and the metabolites in samples from patients under treatment with ibrutinib. Accuracy and precision met the limits as they are described in international guidelines [17,18]. The tool we present here can be used to gain quantitative insight into the extrahepatic metabolism of ibrutinib. For ibrutinib, the correlation between predicted clearance from hepatocyte incubations

and the actual clearance showed a mismatch [8]. As the three thiol metabolites together provide a more complete image of this route, the actual extent of this route can be further evaluated now. This method will be used in the near future to further elucidate the metabolism of ibrutinib through the glutathione cycle. The GSH and CGS metabolites that were previously known appeared only very low-abundant [5]. The in plasma newly discovered ICYS metabolite is the main circulating thiol metabolite in human subjects at trough levels.

Conflict of interest

The authors have no competing interests to declare.

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