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Development and validation of an LC-MS/MS method for the quantification of imatinib and imatinib-d8 in human plasma for the support of an absolute bioavailability microdose trial

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Here we describe the development and validation of an LC-MS/MS method for the quantification of imatinib and imatinib-d8 in plasma for the support of a clinical absolute bioavailability microdosing trial. The focus lies on the technical aspects to analyse high concentrations of imatinib and low concentrations of imatinib-d8 that are present simultaneously in study samples, using a single sample processing and analytical method. With the validated assay, imatinib and imatinib-d8 can be quantified simultaneously in ranges from 25.0 – 5,000 ng/mL and 0.01 – 2.0 ng/mL, respectively. The method was successfully applied in an imatinib-d8 absolute bioavailability microdosing trial, where a 100 µg imatinib-d8 microdose was intravenously administered to a patient on oral imatinib treatment 400 mg once daily.

1. Introduction

Absolute bioavailability is a measurement of the rate and extent to which the active ingredient or active moiety of a drug is absorbed, reaches the systemic circulation and becomes available at the site of action. Absolute bioavailability assessment is an important component to assess during drug development, and results on these trials are increasingly requested by the EMA and FDA for new chemical entities to be able to assess the safety and efficacy of a drug product. (European Medicines Agency, 2019; FDA, 2019)
The conventional way to perform an absolute bioavailability trial is a two-period crossover trial design. By that, patients receive a non-intravenous compound as well as an intravenous compound, both at therapeutic strength, with a washout period in between. By comparing drug exposure following these administrations, the absolute bioavailability can be calculated. An important drawback of this design is that an intravenous formulation needs to be developed and additional pre-clinical safety testing is required.
An alternative way to investigate the absolute bioavailability is by using a microdosing approach. This can be done by administering a therapeutic dose of the drug via the non-intravenous route for intended clinical use, after which a microdose (either radiolabelled drug or stable isotope labeled drug) is given intravenously at 1/100th of the therapeutic dose or less than 100 µg, circumventing the requirement to develop and test the safety of an intravenous formulation (International Conference on Harmonisation, 2009). To be able to quantify plasma drug levels after microdose administration, ultra-sensitive analytical detectors are required. For many years, the only way to perform a microdose trial was by using accelerator mass spectrometry (AMS), where the total amount of ^{14}C in the circulation can be quantified in the attomolar range. (Lappin 2016)
With recent advancement in ultra-sensitive liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) technologies, it is now possible to accurately measure drug concentrations in plasma following intravenous administration of a stable isotope labeled microdose using LC-MS/MS. (Maeda 2011) Therefore, it is no longer required to use ^{14}C-radiolabeled drug as the microdose. This can save time and money, as accelerator mass spectrometry (AMS), which is the analysing technique for ^{14}C-labeled microdoses, is labour- and time-intensive and more costly than LC-MS/MS.
Here we describe the method development and validation of a highly sensitive LC-MS/MS method for the simultaneous quantification of the anticancer drug imatinib and imatinib-d8 in plasma samples for the support of an absolute bioavailability microdose trial in cancer patients treated with the drug. This paper focuses on technical aspects to be able to analyse high concentrations of imatinib (µg/mL range, Fig. 1A) and low concentrations of imatinib-d8 (pg/mL range, Fig. 1B) that are present in the same sample, using a single processing and quantification method.

2. Investigations and results

2.1. Method development

2.1.1. Dynamic range selection

Imatinib is indicated for clinical use in different doses (U.S. Food & Drug Administration, 2001). For the absolute bioavailability trial, patients can be included who have steady state plasma pharmacokinetics at a dose of 400 mg imatinib QD. From previous trials in patients with chronic myeloid leukemia (CML) it is known, that steady state maximum plasma concentrations after 400 mg QD dosing are around 2.6 µg/mL (Peng 2005). The terminal elimination half-life of imatinib is around 19 h, with mean imatinib trough concentrations around 1.2 µg/mL. (Peng 2004). Concentrations in other patient populations, for example with gastro intestinal stromal tumors (GIST), may be lower (Farag 2017). Therefore, a dynamic range from 25 – 5,000 ng/mL was considered sufficient to be able to accurately quantify all imatinib plasma levels after 400 mg imatinib QD dosing during the 24 h dosing interval at steady-state.
For microdose administration in humans, a maximum of 100 µg can be administered as a single intravenous gift. According

to known pharmacokinetic parameters of imatinib, the expected maximum plasma concentration (C_{max}) after administration of an intravenous 100 µg microdose (D) could be calculated using the following formula.

$$Cmax = \frac{D}{Vd} \quad (1)$$

For imatinib, the volume of distribution (V_d) is around 300 L (Peng 2005). The expected C_{max} is therefore around 330 pg/mL after a single intravenous bolus and the C_{48h} around 60 pg/mL. A validated concentration range of 0.01 – 2.0 ng/mL was therefore considered adequate to quantify imatinib-d8 from 0-48h after intravenous administration of a single 100 µg imatinib-d8 intravenous microdose. Using these concentration ranges, the imatinib:imatinib-d8 ratio for all calibration standards and quality control standards was 2500:1.

2.1.2. Selection of stable isotope labeled drug and internal standard

To be able to distinguish orally administered from intravenously administered imatinib using LC-MS/MS, the intravenous drug requires stable isotope drug labeling to increase the molecular mass. The choice of the stable isotope labeled drug was made based on three parameters: expected isotope interference from unlabeled imatinib, location of the stable isotope labels in the imatinib molecule, and commercial availability.

After concomitant administration of a microdose next to a drug that already displays steady state pharmacokinetics, large differences in systemic plasma concentrations between the therapeutic dosed drug and microdosed drug exist. This requires sufficient drug labeling of the microdose, as the unlabeled drug might interfere in higher mass transition channels because of the presence of naturally abundant isotopes. The required amount of drug labels for the imatinib microdose trial was calculated using isotope distribution software (SIS Web). Using the 2,500-fold concentration difference between imatinib and imatinib-d8 in the selected concentration range, it was calculated that a minimum of 6 labels was required to prevent interference from imatinib in the stable isotope imatinib mass transition channel.

When selecting a stable isotope labeled molecule for microdosing, the locations of the labels in the molecular structure are of importance as well. The incorporation of heavier stable isotopes (eg ^{13}C, ^{15}N, ^{2}H) in a drug molecule may result in a change in reaction rate of the drug into metabolites. The presence of one or more stable isotopes incorporated into a drug molecule may therefore result in an altered metabolism as compared to the unlabeled drug, and therefore in an unreliable quantification of the exposure after labeled drug administration. This process, also known as the kinetic isotope effect, is especially relevant for deuterated drugs, where there is an increased bond strength between the carbon and deuterium atoms, which may result in altered pharmacokinetic properties of the labeled drug (Jiang 2012; Sharma 2012) For imatinib, metabolic hot spots were known prior to this study, which made it possible to select a stable isotope labeled drug without any labels on metabolic hot spots in the imatinib molecule (Rochat 2008).

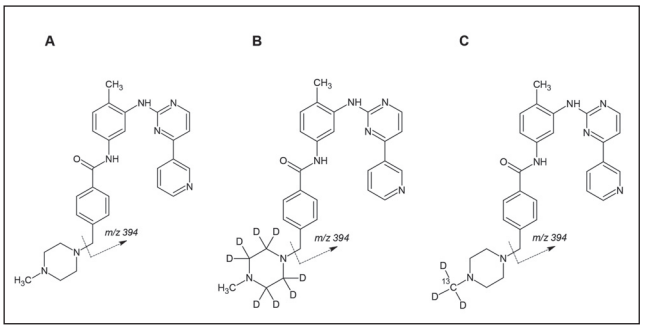

Fig. 1: Chemical structures of (**A**) imatinib, (**B**) imatinib-d8 and (**C**) imatinib -^{13}C,d3, including proposed location of fragmentation

Taking all parameters into account, imatinib-d8 was chosen as the stable isotope labeled molecule of choice, because of the sufficient amount of stable isotope labels, the absence of deuterium labels on metabolic hot spot in the drug molecule, and the fact that is was commercially available at the time of study conception. Imatinib-^{13}C,d3 (Fig. 1C) was selected as an analytical internal standard for both imatinib and imatinib-d8. The concentration of the internal standard was selected to be able to accurately quantify both imatinib and imanitib-d8 concentration levels at the same time, without causing interference in the selected reaction monitoring channels.

2.1.3. Sample pretreatment optimization

Sample pretreatment optimization was initially focused on maximizing the sensitivity for the imatinib-d8 signal, as this is the limiting factor for microdose analyses using LC-MS/MS. By extracting plasma samples with TBME, imatinib and imatinib-d8 could be isolated efficiently and further concentrated by evaporation to reach a lower limit of quantification for imatinib-d8 that fits the purpose of the absolute bioavailability trial. Liquid-liquid extraction also resulted in a low background noise in the imatinib-d8 channel, resulting in an optimal signal-to-noise ratio in the mass spectrometer.

2.1.4. Mass spectrometry

The main technical challenge of the imatinib-d8 absolute bioavailability microdose trial is that high concentrations of imatinib are present in the same sample containing low levels of imatinib-d8. Ideally, both imatinib and imatinib-d8 are quantified following the exact same sample pretreatment and mass spectrometry analysis. Sample pretreatment optimization for imatinib-d8 by concentrating

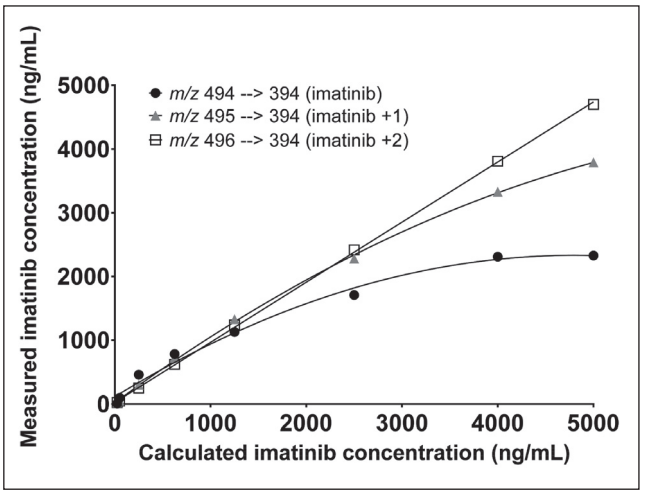

Fig. 2: Linearity of imatinib calibration curves by imatinib isotopologue multiple reaction monitoring. Curves are displayed for imatinib (*m/z* 494 à 394), imatinib +1 (*m/z* 495 à 394), and imatinib +2 (*m/z* 496 à 394)

the final extract results in adequate sensitivity for imatinib-d8, but mass detection saturation for unlabeled imatinib. To solve this issue, we made use of the natural abundant isotopes of imatinib to be able to create a linear calibration range for imatinib as well. The potential of using a less abundant isotope to prevent mass spectrometer detector saturation has successfully been demonstrated in a microdose bioavailability trial in the past (Yuan 2019). As can be seen in Fig. 2, saturation of the mass spectrometer occurred when using the imatinib and imatinib +1 isotope channel, but resulted in a linear calibration curve when using the imatinib +2 isotope channel. The selected mass transition requires two natural abundant isotopes present in the neutral loss fragment of unlabeled imatinib, as depicted in Fig. 1A, and therefore explains the greatly reduced signal in the mass spectrometer (Fig. 3).

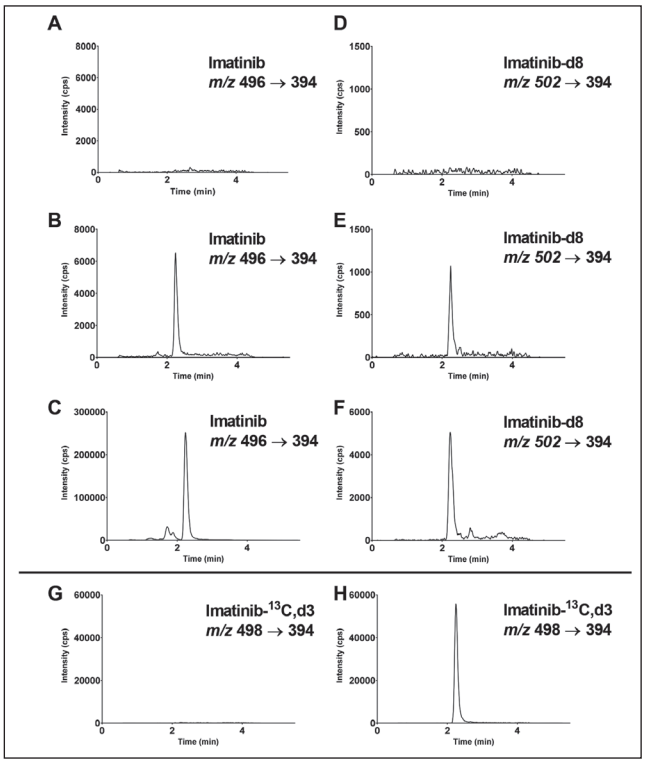

Fig. 3: Representative MRM chromatograms of imatinib in (**A**) blank matrix, (**B**) 25.0 ng/mL calibration standard (LLOQ), and (**C**) 24 h after oral imatinib 400 mg dose, and MRM chromatograms of imatinib-d8 in (**D**) blank matrix, (**E**) 0.01 ng/mL calibration standard (LLOQ), and (**F**) 48 h after intravenous imatinib-d8 100 µg microdose. MRM chromatograms of imatinib-^{13}C,d3 are displayed for (**G**) blank matrix, and (**H**) LLOQ calibration standard

2.2. Validation procedures

2.2.1. Calibration curve

Calibrations standards with a concentration range of 0.01 – 2 ng/mL (imatinib-d8) and 25 – 5,000 ng/mL (imatinib) were prepared in blank human K_2EDTA plasma. Linear regression with a weighting factor of $1/x^2$ was applied, where x equals the concentration of the analyte. The calibration curves were acceptable if 75% of all non-zero calibration standards were within or equal to 15% of the nominal concentrations, or 20% for the LLOQ. These acceptance criteria were met in three separate runs and thus the calibration curves were accepted.

2.2.2. Accuracy and precision

Five replicates of QC LLOQ, QC Low, QC Mid and QC High were prepared and analyzed in three separate runs. The intra-run accuracy, expressed as the bias, was calculated by dividing the difference between the mean measured concentration per run and the nominal concentration by the nominal concentration. The overall bias was calculated similarly, by using the overall mean measured concentration. Intra-run precision, expressed as the coefficient of variation (CV), was calculated by dividing the standard deviation of the measured concentration per run by the mean measured concentration per run. To calculate the inter-run precision a one-way ANOVA was used.

The acceptance criteria were met if the bias and precision values were ±20% and ≤20% at the LLOQ and ±15% and ≤15% for the other tested levels, respectively. As can be seen from Table 1, these criteria were met in three separate runs.

2.2.3. Specificity and selectivity

Six different batches of blank human K_2EDTA plasma were spiked at the LLOQ level and were processed together with double blank samples to assess the selectivity of the assay. The maximum deviation from the nominal concentration was 10.4% for imatinib and 10.5% for imatinib-d8. No interference was detected in the double blank samples for both imatinib, imatinib-d8 and the internal standard, meaning that the selectivity of this assay is sufficient for its intended purpose.

Cross-analyte interferences were tested by spiking blank K_2EDTA plasma separately with imatinib and imatinib-d8 at the ULOQ level and with imatinib-^{13}C,d3 at the concentration used as an internal standard. The interference from imatinib-d8 in the imatinib and imatinib-^{13}C,d3 channel was 11.6% and 1.9%, respectively, and these were considered acceptable since at the LLOQ 20% is allowed. The interference from imatinib-^{13}C,d3 in the imatinib and imatinib-d8 channel was 1.9% for both analytes, and was therefore also considered acceptable.

The cross-analyte interference from imatinib in the imatinib-d8 channel was 270%. At the ULOQ level of imatinib, the interference in the imatinib-d8 channel at the LLOQ level is therefore considered unacceptable. In practice, this 500,000 fold difference between imatinib and imatinib-d8 (5,000 ng/mL vs 0.01 ng/mL) would not occur, as imatinib-d8 is administered at the maximum plasma concentration of imatinib (~2,500 ng/mL), and is from thereon eliminated at the exact same rate, leading to a constant drug ratio of approximately 1:7500 in the systemic circulation. In the absolute bioavailability trial, after 24h, patients will receive a new administration of oral imatinib, while not receiving another intravenous imatinib-d8 microdose. This will lead to a larger difference in the imatinib:imatinib-d8 ratio at the 48h (and last) time-point. For this reason, we investigated the maximum acceptable concentration difference between imatinib and imatinib-d8 that still enables us to accurately quantify imatinib-d8 concentrations. For each imatinib-d8 calibration standard level, imatinib was added at the ULOQ level, and imatinib-d8 concentrations were quantified. The interference per calibration level was calculated, and considered acceptable if a maximum interference of 20% from imatinib in the imatinib-d8 quantification was

Table 1: Assay performance data for the analysis of imatinib and imatinib-d8 in human K_2EDTA plasma

Analyte	Nominal concentration (ng/mL)	N	Intra-assay		Inter-assay	
			Bias (%)	CV (%)	Bias (%)	CV (%)
imatinib	25.0	15	-4.7 – -0.2	1.7 – 6.8	-2.9	1.4
	75.0	15	-0.9 – 2.0	1.8 – 2.7	0.9	1.1
	625	15	-0.4 – 0.8	0.9 – 1.4	0.1	0.3
	3,750	15	-7.5 – -5.1	1.2 – 2.6	-6.3	0.9
Imatinib-d8	0.0100	15	-12.7 – -4.4	8.0 – 9.6	-7.8	2.6
	0.0300	15	-5.6 – -0.9	3.8 – 7.7	-3.6	-*
	0.250	15	-2.4 – -2.0	1.2 – 1.9	-2.2	-*
	1.50	15	-6.4 – -3.7	2.0 – 2.7	-5.4	1.1

*The inter-run precision could not be calculated because there is no significant additional variation owing to the performance of the assay in difference runs

observed. This was the case at an imatinib-d8 concentration level of 0.1 ng/mL (17% interference), leading to the conclusion that at maximum, a 50,000 fold difference (5000 ng/mL vs 0.10 ng/mL) between imatinib and imatinib-d8 in plasma samples is acceptable to be able to accurately quantify imatinib-d8 at the 48h timepoint. As the calibration standards and quality control standards were prepared at a fixed imatinib:imatinib-d8 ratio of 2500:1, this means that patient samples can be accurately quantified as long as the imatinib concentration is between 2,500-50,000-fold higher than imatinib-d8.

2.2.4. *Dilution integrity*

Five replicates of dilution integrity quality control samples were spiked in plasma at concentrations of 7,500 ng/mL and 3 ng/mL for imatinib and imatinib-d8, respectively. Plasma samples were diluted 10 times with blank control plasma. Bias and CV were -5.5% and 1.3% for imatinib and 1.4% and -6.9% for imatinib-d8, meaning all results fell within the acceptance criteria of ±15% and ≤15% for accuracy and precision, respectively. This means that study samples with concentrations above the ULOQ can be diluted 10-times with acceptable accuracy and precision values.

2.2.5. *Lower limit of quantification*

To assess the lower limit of quantification, the absolute signal at the QC LLOQ level was compared to the signal in a blank sample for both imatinib and imatinib-d8, to calculate the signal-to-noise ratio. The signal-to-noise ratio was at least 31 and 12 in plasma for imatinib and imatinib-d8, respectively. This was deemed sufficient as the values were all above the acceptance criterion of 5.

2.2.6. *Matrix effect*

Imatinib and imatinib-d8 were spiked to six different batches of blank human K_2EDTA plasma at QC Low and QC High concentrations and the analyte response in these samples were compared to those in unprocessed samples. The matrix factor was calculated for each batch by calculating the ratio of the peak area in the presence of matrix to the peak area in the absence of matrix. Furthermore, the internal standard normalized matrix factor was calculated. The coefficient of variation of internal standard normalized matrix factor from the six batches was below 1.8% for imatinib and below 3.7% for imatinib-d8. All these values were lower than the acceptance criteria of ≤15%. Therefore, it was concluded that the matrix effect has no effect on the accuracy of the method.

2.2.7. *Carry-over*

Carry-over was assessed by injecting a double blank after the ULOQ. The first double blank response in plasma was less than 10.9% and 19.5% of the mean response of the LLOQ for imatinib and imatinib-d8, respectively, and was considered acceptable.

2.2.8. *Stability*

Short-term stability of imatinib and imatinib-d8 was tested in various matrices under different conditions. An overview of all tested conditions and results is shown in Table 2.

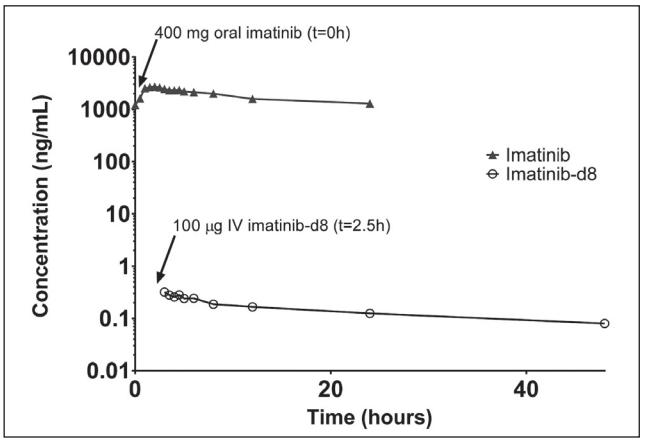

Fig. 4: Plasma concentration-time profile of imatinib and imatinib-d8 following an oral imatinib dose of 400 mg at t=0 h and an intravenous microdose of 100 µg imatinib-d8 at t=2.5 h on the same day in a single patient displaying steady state imatinib plasma pharmacokinetics

Table 2: Stability of imatinib and imatinib-d8 in different matrices under specified conditions

Matrix	Conditions	Analyte	Nominal concentration (ng/mL)	Measured concentration (ng/mL)	Bias (%)	CV (%)	n
Plasma							
Biomatrix	4 freeze-thaw	Imatinib	75.0	76.7	2.3	2.4	3
	cycles		3,750	3,570	-4.7	2.4	3
	(-20°C/Ambient)	Imatinib-d8	0.0300	0.0299	-0.4	4.1	3
			1.50	1.42	-5.6	1.6	3
Biomatrix	72h, Ambient	Imatinib	75.0	75.2	0.3	6.3	3
			3,750	3,570	-4.7	2.9	3
		Imatinib-d8	0.0300	0.0273	-9.0	7.4	3
			1.50	1.41	-6.0	2.6	3
Biomatrix	109 days,	Imatinib	75.0	75.2	0.2	1.8	3
	-20°C		3,750	3,560	-4.9	1.9	3
		Imatinib-d8	0.0300	0.0294	-2.1	3.9	3
			1.50	1.43	-4.9	3.9	3
Final extract	24 days,	Imatinib	75.0	74.1	-1.2	2.0	3
	2-8°C		3,750	3,480	-7.3	2.2	3
		Imatinib-d8	0.0300	0.0291	-2.9	5.3	3
			1.50	1.44	-4.2	2.9	3
NaCl 0.9%	37 days, -20°C	Imatinib	100,000	94,800	-5.2	12.3	3

2.3. Clinical application

The validated assay is used to support a clinical absolute bioavailability microdose trial (EudraCT 2018-003997-28). In this trial, patients who are on steady state plasma concentrations after imatinib 400 mg QD treatment receive a single 100 µg intravenous imatinib-d8 microdose at the estimated T_{max} of imatinib (2.5 h) after oral intake. A plasma concentration-time curve of imatinib and imatinib-d8 after administration of oral imatinib in combination with an imatinib-d8 intravenous microdose is depicted in Fig. 4. Plasma concentrations for both imatinib and imatinib-d8 were in the validated range for all samples analyzed. The concentration difference between imatinib and imatinib-d8 ranged from ~8,200-15,000 fold, resulting in an accurate quantification of imatinib-d8. At the maximum concentration of imatinib (2 h after oral dose), no signal in the imatinib-d8 mass transition channel was observed, providing further confirmation that the validated method is fit for its intended purpose.

3. Discussion

A highly sensitive LC-MS/MS method for imatinib-d8, and imatinib, in human plasma was developed and validated for all validation parameters. The quantifiable range was 25 – 5,000 ng/mL for imatinib and 0.01 – 2 ng/mL for imatinib-d8, based on expected concentrations in a clinical trial. Sample pretreatment consists of liquid-liquid extraction using TBME and concentration of the final extract after sample evaporation and reconstitution. To prevent mass spectrometer detector saturation, a less abundant +2 isotope of imatinib was selected for quantification. For both analytes, imatinib-^{13}C,d3 was used as an internal standard. The method was developed and validated for the support of an absolute bioavailability microdosing trial of imatinib-d8. Clinical applicability was demonstrated in the first treated patient in the trial. Imatinib-d8 can be quantified accurately as long as the concentration difference between imatinib and imatinib-d8 in plasma is between a 2,500-50,000-fold, which was the case for the first patient in the trial.

4. Experimental

4.1. Chemicals and reagents

Imatinib mesylate, imatinib-d8 mesylate, and imatinib-^{13}C,d3 (Fig. 1) were purchased from Sequoia Research Products (Pangbourne, UK), Toronto Research Chemicals (Toronto, ON, Canada), and Alsachim (Illkirch, France), respectively. Acetonitrile (ACN), formic acid (FA), isopropyl alcohol (IPA), methanol (MeOH) and water were of ULC-MS grade and originated from Biosolve Ltd (Valkenswaard, The Netherlands). Ammonium hydroxide (NH_4OH) 25%, dimethylsulfoxide (DMSO) and *tert*-butyl methyl ether (TBME) were from Merck (Darmstadt, Germany). Potassium EDTA (K_2EDTA) plasma was purchased from BioreclamationsIVT (Hicksville, NY, USA).

4.2. Stock solutions, calibration standards and quality control samples

Stock solutions of the analytes (imatinib and imatinib-d8) and internal standard (imatinib-^{13}C,d3) were prepared by dissolution in DMSO, obtaining concentrations of 2.0 mg/mL (imatinib), 0.1 mg/mL (imatinib-d8) and 1.0 mg/mL (imatinib-^{13}C,d3). Working solutions for calibration standards and quality control samples were prepared in DMSO using separate stock solutions. An internal standard working solution was prepared in DMSO as well, resulting in a working solution with a concentration of 10 ng/mL imatinib-^{13}C,d3. Stock solutions and working solutions were stored at -20 °C. Fresh calibration standards were prepared for each validation run by addition of 10 µL of working solution to 190 µL K_2EDTA plasma. Quality control samples were prepared in batches by adding working solution to K_2EDTA plasma in a 1:19 ratio as well, and stored in 200 µL aliquots at -20 °C prior to further processing. Calibration standards and quality control samples contained both imatinib and imatinib-d8 at a 2,500-fold higher concentration for imatinib as compared to imatinib-d8, based on the expected ratios in clinical samples. Concentrations of the calibration standards were 25.0, 50.0, 250, 625, 1,250, 2,500 and 5,000 ng/mL for imatinib and 0.01, 0.02, 0.10, 0.25, 0.50, 1.00, 1.60 and 2.00 ng/mL for imatinib-d8. Quality control samples were prepared at concentrations of 25.0 (QC LLOQ), 75.0 (QC Low), 625 (QC Mid) and 3,750 (QC High) ng/mL for imatinib and 0.01 (QC LLOQ), 0.03 (QC Low), 0.25 (QC Mid) and 1.50 (QC High) ng/mL for imatinib-d8.

4.3. Sample preparation

Prior to processing, samples were thawed and aliquots of 200 µL per sample were transferred to 2.0 mL Eppendorf tubes. To each 200 µL of plasma, 10 µL of internal standard working solution was added, except to double blank calibration standards. Samples were vortex mixed for 10 s after which 1.0 mL of TBME was added for liquid-liquid extraction of the analytes and internal standard. Samples were mixed for 5 min at 1,250 rpm on an automatic shaker before centrifugation at 23,100 g for 5 min. The liquid-layers were separated from each other by snap freezing the samples in an ethanol/dry-ice bath, after which the unfrozen TBME supernatant was transferred to clean 2.0 mL Eppendorf tubes. The supernatant was then dried under a gentle stream of nitrogen at 40 °C using a TurboVap. The dried samples were reconstituted with 100 µL of reconstitution solvent (100 mM NH_4OH:ACN (50:50, v/v) and centrifuged at 23,100 g for 5 min. The clear supernatant was transferred to glass vials with inserts and capped prior to sample analysis.

4.4. Instrumentation and operating conditions

The LC-MS/MS method from a previously validated therapeutic drug monitoring assay, which includes imatinib, was used as a basis for the development and validation of the current assay (Herbrink, 2016). Chromatographic separation was performed on a Phenomenex Gemini C18 column using gradient elution. For detection, a QTRAP 5500 tandem mass spectrometer equipped with a turbo ionspray interface (TIS) was used operating in the positive ionization mode. An overview of the general and analyte specific mass spectrometer settings can be found in Table 3. Parameter settings were optimized for the imatinib-d8 signal, as the sensitivity for the microdose was considered critical for the method to be successfully used to support the absolute bioavailability trial. For imatinib, an isotopologue (+2 Da on the parent mass) was selected to prevent mass spectrometer saturation.

Table 3: General and analyte specific mass spectrometer settings

General settings			
Run duration (min)	5.5		
Ion spray voltage (V)	4000		
Curtain gas (au)	40		
Ion source gas 1 (au)	50		
Ion source gas 2 (au)	50		
Collision gas (au)	9		
Temperature (°C)	500		
Analyte specific setting			
Analyte	**Imatinib**	**Imatinib-d8**	**Imatinib-^{13}C,d3**
Parent mass (*m/z*)	496	502	498
Product mass (*m/z*)	394	394	394
Collision energy (V)	43	43	43
Declustering potential (V)	46	46	46
Collision cell exit potential (V)	42	42	42
Entrance potential (V)	10	10	10

4.5. Validation procedures

A complete validation of the bioanalytical assay for plasma was carried out according to regulatory guidelines (European Medicines Agency 2012; FDA 2018). Validation experiments included calibration curve, accuracy and precision, lower limit of quantification (LLOQ), dilution integrity, carry-over, selectivity, matrix factor and recovery, and stability.

4.6. Clinical application

Clinical applicability of the validated method was demonstrated in plasma samples obtained from a patient in an imatinib-d8 absolute bioavailability microdosing trial, conducted in our institute (EudraCT 2018-003997-28). The treated patient was on oral imatinib treatment (400 mg QD) for at least 7 days prior to study participation to achieve plasma steady state conditions. After hospital admission, the patient received oral imatinib 400 mg QD in the morning, after which a single imatinib-d8 intravenous microdose (100 µg in ~1mL of physiological saline solution) was administered at the estimated maximum plasma concentration of unlabeled imatinib, 2.5 h after the oral dose. Plasma samples were collected at 0 h (pre oral dose), 0.5 h, 1 h, 1.5 h, 2 h, 2.5 h (pre-microdose), 3 h, 3.5 h, 4 h, 4.5 h, 5 h, 6 h, 8 h, 12 h, 24 h and 48 h after oral imatinib dosing.

Conflicts of interest: None declared.

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