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Comparison of ^{19}F-NMR and ^{14}C measurements for the assessment of ADME of BYL719 (Alpelisib) in humans.

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Running Title

The use of 19F NMR to assess ADME of Alpelisib.

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Lists of abbreviations

CRO: Contract Research Organization

DRM: Drug related material

GMP: Good Manufacturing Practice

hADME: Human Absorption, Distribution, Metabolism and Excretion study

HPLC: High performance liquid chromatography

MAS NMR: Magic angle spinning nuclear magnetic resonance spectroscopy

NMR: Nuclear magnetic resonance spectroscopy

LSC: Liquid Scintillation counting

PK: Pharmacokinetics

UPLC: Ultra performance liquid chromatography

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Abstract

The human mass balance study is the definitive study for the assessment of absorption, distribution, metabolism and excretion (ADME) properties of a new chemical entity (NCE) in humans. Traditionally this has been carried out by the administration of radiolabeled drug substances, typically ^{14}C or occasionally ^{3}H, as detection methods for these isotopes allow the absolute quantification of drug related material (DRM) in blood, plasma and excreta. Coupled with the use of analytical techniques such as liquid chromatography-mass spectrometry, a picture of the metabolic fate of a compound can be elucidated. In this study we demonstrate the capabilities of ^{19}F-nuclear magnetic resonance (NMR) spectroscopy, applied as an alternative to radiolabeling, for the determination of mass balance and for metabolite profiling of an orally administered fluorinated drug. To demonstrate the capabilities of NMR, the study was conducted on remaining samples from a ^{14}C human mass balance study conducted on Alpelisib (BYL719), a compound in late stage development at Novartis for the treatment of solid tumors. Quantitative ^{14}C data was used to cross-validate the data obtained by NMR. The data show that, using ^{19}F-NMR, comparable data can be obtained for key human ADME endpoints including mass balance, total DRM determination in plasma and metabolite profiling and identification in plasma and excreta. Potential scenarios where NMR could be employed as an alternative to radiolabeling for the conduct of an early human ADME study are discussed.

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Introduction

Human ADME studies are a standard part of the development process for new drugs. In these studies healthy volunteers or patients are administered the drug containing radiolabel, typically ^{14}C, after which they are hospitalized for a time. During this period, blood (and plasma) samples are taken and excreta are completely collected. The objectives and design aspects of these studies have been extensively reviewed and are not discussed further here (Beumer et al, 2006; Roffey et al, 2007; Nijenhuis et al, 2016). The timing of the human ADME varies (Penner et al, 2012), but general consensus is that the study is done once proof of concept is declared and/or preferably once the phase II dose is known. Ideally, the results should be available before the start of phase III dosing (FDA, February 2008). Having human ADME data during the initial phase 1 studies would be highly advantageous, and would allow for streamlining of later activities. Such an approach was proposed by (Obach et al, 2012). With this strategy, the human ADME (and supporting dosimetry studies) would be the first radiolabeled studies conducted in drug development. Following this, the exposures of major circulating metabolites would then compared to toxicology species using “cold” methodologies, with the result that pre-clinical radiolabeled studies would only be needed in certain circumstances. Despite the attractiveness of the approach, it appears it has not been widely adopted in industry. Possible reasons for this could be the requirements for GMP synthesis of drug products for human use (FDA, July 2008), and the resources needed to prepare for and run a hADME. These studies are costly and time consuming, and there are clear difficulties in convincing company management to move costs to phase 1 of development when compound attrition rates are still high. Instead, the industry has preferred to use “cold” mass spectrometry based approaches to investigate drug metabolites in plasma or blood left over from phase 1 clinical studies, and to establish exposure coverage with toxicology species (Yu et al, 2007; Yi et al, 2010; Ma et al, 2010; Gao et al, 2010). These, or similar, procedures are used extensively across the industry since publication of the FDA MIST guidance (FDA, updated November 2016). The main issue

with mass spectrometry based screening approaches is that, to provide quantitative data, standards of the compounds of interest are needed (it is important to note that for establishing human:animal metabolite exposure ratios, as presented by (Gao et al, 2010), metabolite standards are not required). Leftover radiolabeled samples from early preclinical studies can be used for this purpose, as can chemical or biological synthesis of relevant metabolites following initial screening of the human samples. However these have several drawbacks. Radiolabeled samples from pre-clinical studies may not contain the relevant metabolites, and in any case the trend in industry has been to push these studies as late as possible in development after phase 1 clinical studies are completed. Metabolite synthesis is also often time consuming and costly. Finally, while these approaches address circulating metabolites, they provide little insight into the likely major elimination pathways of the drug.

Another approach that has been used to quantify metabolites in human plasma, and later urine is ^{1}H NMR (Dear et al, 2008, Nedderman et al, 2011). The quantitative power of NMR has been known for several decades but was not used much in drug metabolism studies due to sensitivity limitations. Instrument costs and the need for highly skilled operators were likely also a factor. This has been, in part, addressed by the advent of high field magnets and cryoprobes. ^{19}F NMR has also been used for quantitative metabolite profiling in rat urine and bile (Lenz et al, 2002) and, more recently, to assess mass balance and drug metabolism in pre-clinical ADME studies (Hu et al 2017, Mutlib et al 2012). Fluorine is incorporated frequently into drugs to alter chemical properties, disposition and biological activity (Park et al, 2001), and is compatible with NMR quantitation because of its favorable intrinsic NMR properties including the 100% natural abundance and low background interference.

In this article, we have used samples remaining from a human ADME study conducted with Alpelisib (BYL719) (James et al, 2015) to demonstrate the utility of ^{19}F NMR for the

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determination of key hADME deliverables such as mass balance, quantitative metabolite profiling in plasma and excreta, and determination of total DRM pharmacokinetics (PK) in plasma. The presence of ^{14}C label in the samples was used to cross-validate the ^{19}F NMR results.

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Materials and Methods

Chemicals and standards: [^{14}C]BYL719 was synthesized by the Isotope laboratory of Novartis, Basel, Switzerland (Supplemental data) and was blended with unlabeled BYL719 (Furet et al, 2013) to achieve a specific radioactivity of 6.94 kBq/mg (0.188 µCi/mg). Principle metabolite M4 was supplied by Novartis, Basel, Switzerland (Supplemental data). The chemical structures/formula of ^{19}F-NMR internal standards 1 (Fevipiprant, supplied by Novartis, Basel Switzerland (Sykes et al, 2006; Bala et al, 2005)) and 2 (obtained from commercial sources) are displayed in Table 1. All other chemicals and solvents were of the highest analytical grade available and were obtained from commercial sources.

Human ADME study with BYL719: Plasma, urine and feces samples used for the ^{19}F-NMR investigations were obtained from an open label, oral dose ADME study where BYL719 was administered to four healthy male subjects All subjects provided written informed consent prior to enrollment. The study followed the ethical principles of the Declaration of Helsinki and the ICH Harmonized Tripartite Guidelines for Good Clinical Practice and applicable local regulations (European Directive 2001/20/EC and US Code of Federal Regulations Title 21) and was approved by an independent ethics committee prior to site initiation. The clinical part of the study was conducted at PRA Intl, Zuidlaren, the Netherlands. Each subject received a single oral dose of 400 mg [^{14}C]BYL719 in two gelatin capsules of 200 mg [^{14}C]BYL719 each (total dose: 2.78 MBq, 75 µCi). Subjects were hospitalized for up to eight days, during which time blood, plasma, urine and feces were collected. Total radioactivity determination in blood, plasma, urine samples and feces homogenate were made by LSC measurements conducted in the bioanalytical laboratory of the study site. Further details of this clinical study and results are published elsewhere (James et al, 2015).

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*Radioactivity determination in excreta***:** Radioactivity contents in urine and feces homogenates were determined at the bioanalytical laboratory of PRA Intl., Zuidlaren, the Netherlands. These data were used for the comparison with ^{19}F NMR data for the determination of mass balance. For urine, duplicate (1000 µL) aliquots were placed into 7 mL glass vials (Perkin Elmer), after which 5 mL of scintillation cocktail (Ultima Gold^{TM}, Perkin Elmer) was added. After vortex mixing for at least 5 seconds, each sample was placed in a Tri-Carb^{TM} 3100 TR liquid scintillation analyzer (Waltham, MA, USA) at least 30 minutes before counting. The total ^{14}C-radioactivity of the samples was determined by counting until a statistical error (2s) of 0.5% was obtained with a counting time of 10 minutes.

For feces homogenates, quadruplicate, accurately weighed (500 mg) aliquots were dried in a stove at 50°C for at least three hours. After the addition of 100 µL combusto aid (Perkin Elmer) to the dry homogenates, the samples were combusted in a sample oxidizer model 307 (Perkin Elmer). 7 mL CarboSorb-E (Perkin Elmer) was used as absorber agent for carbon dioxide. At the end of the combustion cycle the absorber was mixed with 13 mL of the scintillant PermaFluor E. The samples were placed in the liquid scintillation analyzer for at least 60 minutes before counting. The total ^{14}C-radioactivity of the samples was determined by counting until a statistical error (2s) of 0.5% was obtained with a counting time of 10 minutes.

Radioactivity determination in plasma and plasma extracts: Aliquots of plasma were solubilized in a mixture of Soluene 350 (Packard)/Isopropanol (2:1 v:v). After complete dissolution, the samples were neutralized with Hydrochloric acid (2M) and mixed with Irgasafe-Plus liquid scintillation cocktail (Zinsser Analytic, Maidenhead, Berkshire, UK) for liquid scintillation counting. Aliquots of plasma extracts were measured directly in either 20 mL or 6 mL antistatic polyethylene vials (Packard BioScience, Groningen, The Netherlands) containing 5 mL Irgasafe-Plus liquid scintillation cocktail. All samples were assayed for ^{14}C-radioactivity in a LSC counter model Tri-Carb 2200CA using an external standard ratio method for quench correction.

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Preparation of calibration samples for quantitative ^{19}F NMR analysis: For the quantification of total DRM in plasma and excreta by ^{19}F NMR, and for quantitative metabolite profiling by ^{19}F NMR, calibration curves were prepared. A stock solution of BYL719 was prepared by solubilization of a weighed amount in CD_3OD/D_2O (7:3 v/v), containing either internal standard 1 or 2 at a set concentration. Calibration samples were subsequently prepared by serial dilution of this stock solution in CD_3OD/D_2O (7:3 v/v), containing either internal standard 1 or 2 at a set concentration.

Preparation of individual plasma timepoints for analysis of total DRM by ^{19}F-NMR: From one subject, 3 mL of plasma was taken from timepoints 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 24 and 48 hours post administration of the dose and each was placed into a separate 50 mL BD Falcon tube. The plasma aliquots were subsequently extracted as follows: To each sample, 6 mL of ice cold Acetonitrile was added and the resulting suspension was vortex mixed for 30 seconds and subjected to ultrasound (ultra-sonication) for 5 minutes in a water bath. This was followed by centrifugation (10 min, 8331 × g; GS-15R centrifuge, Beckman), after which the supernatant was removed from the pellet. The pellet was re-suspended by the addition of 1 mL of water, followed by vortex mixing (30 seconds) and ultra-sonication (5 min). To this, 8 mL of ice cold Acetonitrile was added and the mixture was further vortex mixed (30 seconds) and subjected to ultra-sonication (5 minutes). Finally the mixture was centrifuged as described above and the resulting supernatant was combined with the first. The resulting pellet was re-extracted a further two times, following the procedure described above. The final combined supernatant was evaporated under a stream of nitrogen at room temperature to a volume of approximately 1 mL, and was transferred to a 2 mL polypropylene tube. The original 50 mL Falcon tube was rinsed twice with acetonitrile, and these rinses were combined with the rest of the extract. Finally, the extract was evaporated to dryness under Nitrogen flow at room temperature.

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Each extract was re-constituted by the addition of 700 µL CD_3OD/D_2O (7/3 v/v) which contained internal standard 1 at a concentration of 5 µg/mL. 170 µL of each reconstitute was transferred to a 3 mm NMR tube and was submitted for analysis.

Preparation of plasma AUC pool for quantitative metabolite profiling: For one subject, volumes of plasma from timepoints 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 hours post administration of the dose were pooled in order to mimic an area under the curve (AUC) exposure, as previously described (Hamilton et al, 1981). The total pool volume was 8832 µL. The pool was split into 8 polypropylene tubes (15 mL capacity), with each containing approximately 1.1 mL of plasma. The plasma samples were extracted as follows:

To each sample, 2.2 mL of ice cold Acetonitrile was added and the resulting suspension was vortex mixed for 30 seconds and subjected to ultrasound (ultra-sonication) for 5 minutes in a water bath. This was followed by centrifugation (10 min, 8331 × g; GS-15R centrifuge, Beckman), after which the supernatant was removed from the pellet. The pellet was re-suspended by the addition of 0.3 mL of water, followed by vortex mixing (30 seconds) and ultra-sonication (5 min). To this, 2.2 mL of ice cold Acetonitrile was added and the mixture was further vortex mixed (30 seconds) and subjected to ultra-sonication (5 minutes). Finally the mixture was centrifuged as described above and the resulting supernatant was combined with the first. The resulting pellet was re-extracted a further two times, following the procedure described above. The supernatants were combined from all plasma aliquots and the resulting solution was evaporated under a stream of nitrogen at room temperature to a volume of approximately 4 mL. This was transferred to a 10 mL glass tube. The original tube containing the combined supernatants was rinsed three times with water/acetonitrile (9/1 v/v), and these rinses were combined with the rest of the extract. After removal of an aliquot (400 µL) for LSC, the remainder was injected onto the prep-HPLC system and fractionated as described below. Resulting dried fractions were reconstituted by the addition of 600 µL of CD_3OD/D_2O (7/3 v/v)

which contained internal standard 2 at a concentration of 2 µg/mL, followed by vortex mixing and ultra-sonication. The resulting solutions were transferred into 5 mm NMR tubes and were submitted for ^{19}F NMR analysis

*Preparation of urine and feces samples for analysis of total DRM by ^{19}F NMR***.** From all subjects in the study, at time periods pre-dose, 0-6 h, 6-12 h, 12-24 h, 24-48 h, 48-72 h and 72-96 h, duplicate 2 mL aliquots of urine were weighed into 5 mL polypropylene tubes. In parallel, duplicate 0.4 mL aliquots were taken for determination of radioactivity by LSC. The 2 mL aliquots were dried under a stream of Nitrogen at room temperature, and were reconstituted by the addition of 1 mL CD_3OD/D_2O (7/3 v/v) which contained internal standard 1 at a concentration of 25 µg/mL. Reconstituted samples were vortex mixed (10 seconds), ultra-sonicated (30 min) and vortex mixed again for a further 10 seconds. Two aliquots of 150 µL were taken for LSC analysis and 650 µL was transferred into 5 mm NMR tubes for ^{19}F NMR analysis.

From all subjects in the study, at time periods pre-dose, 0-24 h, 24-48 h, 48-72 h, 72-96 h, 96-120 h, 120-144 h and 144-168 h, about 0.2 mL aliquots (duplicate) of fecal homogenate were taken for determination of radioactivity by LSC. In parallel triplicate 1 g aliquots of feces homogenate were weighed into 15 mL polypropylene tubes. The 1g aliquots were extracted as follows: To each sample, 5 mL of ice cold Acetonitrile was added and the resulting suspension was vortex mixed for 10 seconds and subjected to ultrasound (ultra-sonication) for 30 minutes in a water bath. This was followed by centrifugation (10 min, 10000 × g; Allegra® 64R centrifuge, Beckman Coulter), after which the supernatant was removed from the pellet. The pellet was re-extracted by the addition of 1 mL of acetonitrile, followed by vortex mixing, ultra-sonication and centrifugation as described above. After removal of the supernatant, the pellet was extracted a third time in an identical manner. Supernatants from each extraction step were combined and evaporated to dryness under Nitrogen. Samples were reconstituted by the addition of 1 mL

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CD_3OD/D_2O (7/3 v/v) which contained internal standard 1 at a concentration of 25 µg/mL. Reconstituted samples were vortex mixed (10 seconds), ultra-sonicated (30 min) and vortex mixed again for a further 10 seconds. Two aliquots of 150 µL were taken for LSC analysis and 650 µL was transferred into 5 mm NMR tubes for ^{19}F NMR analysis.

Preparation of urine and feces pools for quantitative metabolite profiling: Urine was pooled across the time range 0-144 h by taking equal percentages of the amount excreted from each time period (Subject 1000_00010). After removal of duplicate aliquots (0.5 mL) for LSC, 8 mL of urine was directly injected onto the prep-HPLC system and fractionated as described below. Resulting dried fractions were reconstituted by the addition of 600 µL of CD_3OD/D_2O (7/3 v/v) which contained internal standard 2 at a concentration of 2 µg/mL, followed by vortex mixing and ultra-sonication. The resulting solutions were transferred into 5 mm NMR tubes and were submitted for ^{19}F NMR analysis.

Feces homogenate was pooled across the time range 0-216 h by taking equal percentages of the amount excreted from each individual time period (Subject 1000_00010). After removal of duplicate aliquots (~0.2 g) for LSC, a sample of ~1 g was weighed into a 15 mL polypropylene tube. The sample was extracted as described previously for the analysis of total DRM by ^{19}F NMR. Following evaporation to dryness under N_2, The extraction residue was reconstituted by the addition of 4 mL of water, followed by vortex mixing (2 minutes) and ultra-sonication (15 min). This reconstitute was transferred to a Waters Autopurification system injection tube, after which the original 15 mL polypropylene tube was rinsed twice by the addition of 2 mL of water. Rinses were combined with the original reconstitute. Following removal of duplicate aliquots of 100 µL for LSC, the remaining reconstitute was directly injected onto the prep-HPLC system and fractionated as described below. Resulting dried fractions were reconstituted by the addition of 600 µL of CD_3OD/D_2O (7/3 v/v) which contained internal standard 2 at a concentration of 10

µg/mL, followed by vortex mixing and ultra-sonication. The resulting solutions were transferred into 5 mm NMR tubes and were submitted for ^{19}F NMR analysis.

Semi-preparative HPLC conditions for fractionation of plasma extract, urine and feces extract for quantitative metabolite profiling by ^{19}F NMR: Semi-preparative HPLC with fraction collection for quantitative metabolite profiling was carried out using a Waters Autopurification System equipped with MassLynx™ and the FractionLynx™ application manager. The column used was a Waters Atlantis Prep T3 (10 × 150 mm, 5 µm particle size), at room temperature. Post column flow was directed to a Gilson FC204 fraction collector operated in a time-slice mode. Separation and fractionation of BYL719 metabolites was accomplished using 10 mM ammonium acetate adjusted to pH 5.0 with acetic acid as mobile phase A and Acetonitrile as mobile phase B. Flow rate was 5 mL/min. The semi-preparative HPLC gradient was as follows: Initial conditions were 10% mobile phase B, which increased to 70% at 10 minutes post injection. From there, mobile phase B was increased to 95% at 11 minutes, where it was held (isocratic) up to 15 minutes. Finally, mobile phase B was decreased to 10% at 15.5 minutes, after which the column was re-equilibrated for subsequent injections. Post column flow was fractionated at 12 seconds per fraction into a 96 deep-well plate (Nunc 278752 U96 DeepWell 96-Well x 2mL Assay Collection And Storage Microplate Without Lid, Round Bottom Wells, Non-Treated Natural Polypropylene). Individual fractions were subsequently prepared for ^{19}F NMR analysis as described above.

NMR instrument parameters used for the measurement of total ^{19}F in urine, feces extracts and semi-preparative HPLC fractions: 1D ^{19}F spectra were acquired at a temperature of 300 K using a Bruker 600 MHz Avance III NMR spectrometer equipped with a 5 mm ^{1}H/^{19}F-^{13}C/^{15}N/D CryoProbe™ with a z-gradient system. A total of 128 scans were accumulated for each sample using a standard proton inverse-gated decoupling pulse sequence with a relaxation delay of 7 sec. 32768 complex points covering 34090.9 Hz were recorded at a transmitter frequency

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offset of -75 ppm. Data were zero-filled to 65536 complex points prior to Fourier transformation and an exponential window function was applied with a line-broadening factor of 1.0 Hz. The spectra were manually phase and baseline corrected and referenced to either the internal standard Fevipiprant (= –61.0 ppm) or 3,5-Bis(trifluoromethyl)phenol (= – 64.0 ppm). Based on these parameters the total acquisition time was approximately 15 minutes per sample.

LC-(MS/MS) conditions for identification of drug related components observed following ^{19}F NMR analysis of prep-HPLC fractions: LC-(MS/MS) of prep-HPLC fractions with detectable ^{19}F NMR signals was carried out either using an Agilent model 1200 HPLC coupled with a Waters Synapt HDMS or a Waters UPLC system coupled with a Waters Synapt-G2 HDMS. The column used in both cases was a Waters Atlantis dC18 (2.1 × 150mm, 3µm) equipped with a pre-column of the same type (2.1 × 10mm), which was placed in a column oven at 40°C. Mobile phase A was 10 mM ammonium acetate (aq) adjusted to pH 5.0 with acetic acid. Mobile phase B was Acetonitrile. Flow rate was 0.25 mL/min. The HPLC gradient was as follows: Initial conditions were 30% mobile phase B, which increased to 70% at 10 minutes post injection. From there, mobile phase B was increased to 100% at 11 minutes, where it was held (isocratic) up to 17 minutes. Finally, mobile phase B was decreased rapidly to 30%, after which the column was re-equilibrated for subsequent injections.

Assessment of potential matrix effect on the measurement of total ^{19}F in plasma extracts, urine and feces extracts: To assess any potential matrix effect on the ^{19}F NMR measurement, a series of samples were prepared in control human urine, feces extract, plasma extract and NMR solvents (CD_3OD/D_2O 7/3 v/v). In the case of urine, two aliquots of urine were evaporated to dryness under N_2. For feces, two ~1 g aliquots were extracted as previously described. For plasma, two ~3 g aliquots were extracted as previously described. The resulting residues were reconstituted in CD_3OD / D_2O (7/3 v/v). The reconstitution solvent contained sufficient BYL719

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and Internal standard 2 to achieve, for the first aliquots, a concentration of 100 µg/mL and 25 µg/mL, respectively. The final concentrations in the second aliquots were 1 µg/mL and 2.5 µg/mL, respectively. A control sample without matrix (70/30 v/v CD_3OD/D_2O) was also prepared at both sets of concentrations. The final solutions were placed into 5 mm NMR tubes and were analyzed as described above. Measurements were carried out in quintuplicate in order to assess both the effect of the matrix and NMR instrument precision at the two BYL719 concentrations.

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Results

Excretion balance: The percentage of dose excreted in each collection interval, for both urine and feces, and comparing ^{19}F NMR and ^{14}C measurements are shown in Figure 1. For urine, the percentage of dose excreted up to 96 hours post dose was 13.4 ± 3.83%, based on total ^{14}C measurement, and 12.3 ± 2.47% when measured by ^{19}F NMR. For feces, up to 168 hours post dose, 80.9 ± 3.24% of the dose was excreted (^{14}C measurement) compared to 83.7 ± 3.95% when measured by ^{19}F NMR. With both means of measurement, excretion of the administered dose could be classified as complete (>90%). Good agreement between ^{19}F and ^{14}C measurements was obtained for all collection intervals for both urine and feces.

Total DRM in collected plasma samples: The concentrations of total DRM in plasma extract from 0.5 to 12 hours, determined by ^{19}F-NMR analysis and compared with total ^{14}C measurements of the same plasma extract are detailed in Figure 2. 48 hours after dosing, total DRM was below the limit of quantification by both ^{19}F NMR and ^{14}C measurements. At 24 hours after dosing, concentrations were measureable by ^{14}C but not by ^{19}F NMR. In our conditions, the limit of quantification by ^{19}F NMR was estimated at 500 ng eq/mL for BYL719. The LOQ for the plasma analysis by LSC, which is dependent on the specific activity of the administered drug and the validated ranges of the instrumentation, was approximately 75 ng eq/mL for this study. Some variability was observed between the ^{19}F NMR and ^{14}C measurements (with maximum 19% difference and 3 samples above 10%). As a final step, AUC0-12h was calculated based on NMR and ^{14}C measurements. The results were comparable, showing a 9% difference.

Metabolite profiling in urine: ^{19}F NMR analysis of individual fractions generated from the analysis of the 0-144h urine pool allowed reconstruction of a chromatogram (Figure 3). Three peaks were observed. Subsequent analysis by LC-(MS/MS) showed that the early eluting peak (P1) was comprised of co-eluting metabolites M1 and M12, the middle peak (P2) metabolites M3, M4

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and M9 and the last peak (P3) BYL719. Structures of all metabolites observed in this study are shown in Figure 6. ^{19}F mass balance measurements indicated that 12.6% of the dose was excreted in urine over this time interval. Therefore, in terms of % of dose, 2.31% was attributed to BYL719, 9.43% to M3, M4 and M9, and 0.87% to M1 and M12. This compared well to results obtained with conventional ^{14}C radioprofiling and mass balance measurements, where BYL719 represented 2.04%. M3, M4 and M9 9.15% and M1 and M12 1.15% of the dose. Notably, metabolites co-eluted under the preparative HPLC conditions used to prepare samples for ^{19}F NMR, but were resolved with the HPLC method used in the original human ADME. This is discussed further below.

Metabolite profiling in feces: ^{19}F NMR of individual fractions generated from analysis of a 0-216h feces pool allowed reconstruction of a chromatogram (Figure 3). Four peaks were observed (P1, P2, P3 and P4). Subsequent analysis by LC-MS/MS showed P1 consisted of metabolite M12, P2 consisted of co-eluting M2 and M8, P3 consisted of co-eluting M3, M4 and M9 and P4 was attributed to BYL719. ^{19}F mass balance measurements indicated that 85.9% of the dose was excreted over this time interval. Therefore, in terms of percentage of dose, 0.79% was attributed to M12, 1.59% to M2 and M8, 41.4% to M3, M4 and M9 and 42.1% to BYL719. In the original human ADME, results from ^{14}C radioprofiling were slightly different, with 0.713%, 1.28%, 32.3% and 32.0% of the dose being attributable to the groups of metabolites, respectively. All metabolites detected in the human ADME, with the exception of a minor component M19 (0.5% of dose) were detected by ^{19}F NMR.

Metabolite profiling in plasma: ^{19}F NMR of individual fractions generated from analysis of a 0-24h pool allowed reconstruction of a chromatogram (Figure 4). Two compounds were detected; M4, which represented 23.9% and Alpelisib which represented 76.1% of AUClast. ^{14}C radioprofiling of individual plasma timepoints from 0-12h from the same subject showed M4

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representing 28.8% and Alpelisib 63.7% of AUClast. Two minor metabolites, M3 and M12, which represented 0.13% and 1.33% of the ^{14}C AUClast, respectively, were not detected with the ^{19}F NMR method. Using the total DRM in each plasma sample derived from ^{19}F NMR measurements, an AUC0-12h of 28869 nM*h was calculated. Considering the ^{19}F chromatogram, this translates to an AUC0-12h of 6900 nM*h for metabolite M4 and 21969 nM*h for BYL719. In the original human ADME, the derived AUC0-12h were 8990 nM*h and 19900 nM*h for metabolite M4 and BYL719, respectively. It is important to note that in the case of the ^{14}C derived parameters the presence of additional minor metabolites M3 and M12, and ^{14}C lost during sample processing were taken into account in the calculation.

Assessment of matrix effect on NMR measurements: The NMR integrals (relative to internal standard) for BYL719 spiked into urine, feces extract, plasma extract and NMR reconstitution solvent are shown in Figure 5. The relative standard deviations are calculated from n=5 measurements. The final concentrations of BYL719 in the NMR tubes were 100 µg/mL and 1 µg/mL, respectively. From the data, it is clear that the matrix had negligible impact on the ^{19}F NMR signal under our analytical conditions.

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Discussion

NMR is/has been used for a number of applications in biomedical research and development, including metabolomics, in vivo magnetic resonance spectroscopy and pharmaceutical analysis (drug identification, drug impurity characterization, degradation studies, drug isomeric composition and analysis of counterfeits (Holzgrabe, 2010; Malet-Martino and Holzgrabe, 2011, J.R. Everett et al, 2015). Quantitative NMR has also been used for drug metabolism investigations, such as quantification of biologically isolated metabolites for use in pharmacological activity testing (Mutlib et al, 2011), as standards for quantitative assays to address MIST guidance concerns (Espina et al, 2009, Walker et al, 2011) or in preclinical *in vivo* efficacy models (Walker et al, 2014). It has also been used in pre-clinical *in vivo* studies to establish the urinary metabolic fate of 2-bromo-4-trifluoromethylaniline in rat (Scarfe et al, 1998; Scarfe et al, 1999) and to conduct ADME in pre-clinical species (Mutlib et al, 2012). In human studies it has been applied to urinary mass balance and metabolite profiling (Skordi et al, 2004; Nedderman et al, 2011) and quantitative metabolite profiling in plasma (Dear et al, 2008). In these examples either ^{1}H or ^{19}F NMR was used. ^{19}F has advantages for the quantification of drugs and metabolites in complex biological matrices, due to the extremely low level of endogenous fluorine containing compounds (Martino et al, 2005). Fluorine is incorporated frequently into drugs to alter physicochemical properties (Park et al, 2001), which makes ^{19}F NMR potentially applicable for the study of drug ADME.

In this study, we investigated whether ^{19}F NMR could be used to achieve the following hADME objectives; i) determination of mass balance, ii) determination of total DRM PK in plasma, iii) quantitative metabolite profiling in plasma (MIST guidance considerations), and iv) quantitative metabolite profiling in excreta to assign the major metabolic elimination pathways. Using leftover samples from the human ADME study with Alpelisib allowed for cross-validation of the NMR data with radioactivity data. Excellent agreement was obtained between ^{19}F and ^{14}C

measurements for all of the listed objectives. Nevertheless, our experience conducting the study raised several points that require additional discussion.

NMR quantitation: Quantification using NMR can be accomplished using internal or external standards (Holzgrabe, 2015). For quantification the internal reference method was chosen for this study, in order to assure the highest accuracy and precision (Cullen et al, 2013, Giraudeau et al, 2014). Key considerations that need to be taken into account when selecting a suitable internal standard are; i) the fluorine signal doesn't overlap with those of the drug and metabolites ii) the signal intensity should not differ more than 50-fold from the expected lowest and highest concentrations and iii) to avoid off-resonance effects the fluorine shift should not differ too much from the analytes of interest (Power et al, 2016). For fluorine NMR, use of an appropriate internal standard for a specific chemical shift region should not present problems as a wide range of compounds could be used. In our study we selected either Fevipiprant or 3,5-Bis(trifluoromethyl)phenol as internal standards, which were added to every sample. In Figure 7 a typical 1D ^{19}F-spectrum is shown, revealing the signals from the internal standard and Alpelisib + metabolites in human feces extract. Quantification is based on signal intensity as indicated in the figure. Although no evidence of matrix effects were observed in this study, it is known that matrix can have an impact on NMR quantitation, such as salt concentration. Even though high salt concentrations result in "lossy samples" due to increased conductivity, this does not have any effect on relative signal integrals, since analytes and internal standards are equally effected (Robosky et al, 2007; Voehler et al, 2006). To minimize any additional matrix effects calibration curves can be recorded with the standards being prepared in the matrix of interest. In order to avoid signal saturation effects, T1 relaxation times of the parent compound and the internal standard were determined and the relaxation delay adjusted accordingly.

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Mass balance: Measurement of mass balance relies on complete collection of excreta. If a ^{19}F NMR approach is considered for a phase 1 study, collection of feces needs to be added to a cohort in the single ascending dose arm of the study. This is not commonly done, and key questions that need to be asked are; i) does the clinical unit selected have experience with feces collection and ii) how will the feces be processed? Internal experience suggests that it is difficult to convince teams to agree to feces collections for more than 5 days in a phase 1 study, despite limited additional cost, but it is critical for this approach that it's possible to collect longer (minimum 7 days). Processing of stools can either be done at the clinical site, in the analytical laboratory of the sponsor company, or can be outsourced to a second CRO familiar with feces homogenization procedures. From an analytical perspective, measurement of mass balance in urine is relatively simple, with minimal sample processing required. For feces an organic solvent extraction needs to be performed to prepare samples for NMR analysis. Low extraction recovery, which cannot be measured using this approach, would contribute to low mass balance. The use of high-resolution magic angle spinning (HR-MAS) NMR may compensate for this as feces homogenate could be loaded directly into the sample tube for measurement. HR-MAS NMR is a technology especially suitable for viscous or semi-solid samples. It combines the advantages of sensitive high-resolution NMR probes with magic angle spinning technology for suppression of dipolar couplings known from solid state NMR. It has successfully been applied to a wide range of inhomogeneous samples like gels, swollen polymers, resins, foodstuff, cells and tissue samples (Power, 2011). However, the applicability for quantification of low-molecular weight compounds in feces remains to be tested.

Total DRM PK: In this study, total DRM in individual plasma timepoints was measured by ^{19}F NMR. As for feces, plasma samples were extracted before analysis which could lead to possible losses. A combination of lyophilization followed by HR-MAS NMR may account for this, as discussed above. However this would likely only allow measurement of "free" compound

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related material that was not extractable by protein precipitation. Covalently bound material, e.g. to plasma proteins, would probably not be quantifiable due to broadening of ^{19}F resonances.

Metabolite profiling: We have demonstrated that ^{19}F NMR can provide reliable quantitative metabolite profiles in plasma and excreta for the assessment of metabolite exposures and metabolic elimination pathways. As previously discussed, extraction recoveries cannot be measured for plasma and feces. This is the most likely explanation for the discrepancies that were noted in the ^{19}F and ^{14}C profiles for feces as, in the latter case, column and extraction recoveries were taken into account in the calculation. A second limitation was co-elution of metabolites in the excreta profiles. Development of chromatography methods that separate all metabolites of interest is more challenging with a preparative HPLC system. These limitations could be overcome by, for example, multiple repeated injections with fraction collection using a HPLC/UPLC system. In addition, co-elution may not be an issue for some compounds. Fluorine resonances are very sensitive to structural and steric changes so it is feasible that a mixture of co-eluting metabolites could be quantified individually (Hu et al, 2017). Finally, the fluorine group may be removed by metabolism. Although unlikely for a CF_3 moiety, it is known that single fluorine atoms in a drug can be removed by oxidative defluorination (Park *et al* 2001). This would make the metabolite unavailable for NMR quantification.

Overall summary: We have demonstrated that hADME objectives are achievable using ^{19}F NMR. The availability of complementary ^{14}C data demonstrates that the NMR data can be of high quality, despite the limitations discussed above. The main advantage of this approach is that it could be applied in a phase 1 clinical study without the need to use radiolabel and with limited additional cost. A “human first” approach would allow for streamlining of follow up activities such as animal radiolabeled ADME studies, DDI *in vitro* and clinical studies and defining regulated bioanalysis strategy in toxicology and clinical studies to answer the key

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questions raised by the human data. Furthermore, ^{19}F NMR provides an opportunity to quantitatively investigate metabolite systemic exposures at steady state, as requested by the MIST guidance. Multiple dosing of a radiolabeled tracer is not practical which means investigation of steady state levels typically relies on, for example, development of validated bioanalytical assays or extrapolations from single dose data.

The need for a traditional ^{14}C human ADME study later in development could also be questioned, potentially saving the need to expose healthy volunteers to a radioactive dose of the drug. The main limitations of the study design are sensitivity of the NMR instrument and that it is only applicable to fluorinated drugs. Whether ^{19}F NMR would be sensitive enough to apply to a given clinical study is complex to answer. The dose of administered drug, the extent of its metabolism, the extent of drug absorption and distribution and the rate of excretion are all factors that should be considered. Finally, the number of fluorine atoms (CF_3 vs single F) will also influence the sensitivity as CF_3 resonances are equivalent and give rise to a 3 fold more intense signal. Available PK, TK and pre-clinical ADME data can be used, in addition to the proposed clinical dose, to evaluate the likelihood of success. In our experience, we estimate that, for an orally dosed drug containing a CF_3 moiety, a ^{19}F NMR study could be considered for doses above 50 mg provided factors discussed above are also taken into consideration.

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Authorship contributions

Participated in research design: James, Marvalin, Luneau, Meissner and Camenisch

Conducted experiments: Marvalin and Luneau

Performed data analysis: James, Marvalin and Luneau

Wrote or contributed to the writing of the manuscript: James, Marvalin, Luneau, Meissner

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Figure legends

Figure 1: Mean cumulative excretion of dose in urine and feces after a single oral dose of 400 mg [^{14}C]BYL719 (alpelisib) to four healthy volunteers, determined by liquid scintillation counting and ^{19}F NMR.

Figure 2: Concentrations of total DRM in individual plasma timepoints from a single healthy volunteer after a single oral dose of 400 mg [^{14}C]BYL719 (alpelisib), determined by liquid scintillation counting and ^{19}F NMR. Integration of ^{19}F NMR signals was performed manually.

Figure 3: Reconstructed ^{19}F NMR chromatogram showing the metabolic profile of BYL719 (Alpelisib) in A (urine) and B (feces). Chromatograms reconstructed from total ^{19}F NMR quantification in individual fractions generated by time-slice fraction collection on a preparative-HPLC system. Metabolites were identified by LC-MS/MS analysis of fractions containing ^{19}F. The percentages listed are the percentage of administered dose attributable to the components, determined by ^{19}F / ^{14}C.

Figure 4: Reconstructed ^{19}F NMR chromatogram showing the metabolic profile of BYL719 (Alpelisib) in a plasma AUC0-24h pool of a single subject. Chromatogram reconstructed from total ^{19}F NMR quantification in individual fractions generated by time-slice fraction collection on a preparative-HPLC system. Metabolites were identified by LC-MS/MS analysis of fractions containing ^{19}F. The percentages listed are the percentage of total observed DRM in the sample, determined by ^{19}F / ^{14}C.

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Figure 5: Chart showing the internal standard corrected ^{19}F NMR integrals obtained from analysis of standard solutions of BYL719 (alpelisib) and internal standard 2, spiked into urine, feces, plasma and NMR solvents at analyte:Internal standard concentrations of 100:25 and 1:2.5 µg/mL, respectively. Each sample was analyzed a total of 5 times by ^{19}F NMR. Relative standard deviations from the 5 measurements are listed.

Figure 6: Structures of metabolites of Alpelisib (BYL719) identified in human plasma, urine and feces.

Figure 7: Excerpt from typical 1D ^{19}F-spectrum showing the signal of the internal standard Fevipiprant and of Alpelisib + metabolites. The spectrum was acquired on a 24-48h human feces extract. The major metabolites of Alpelisib were not resolved from the parent compound in the ^{19}F NMR spectrum.

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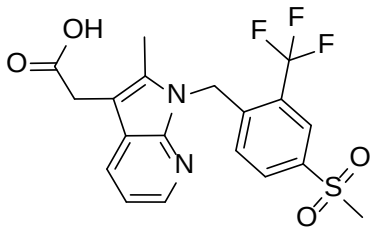
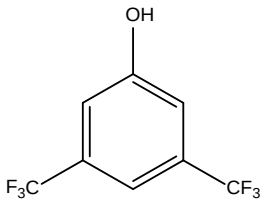
Tables

Table 1. Details of internal standards used for ^{19}F NMR quantification.

Standard ID	Internal standard 1	Internal standard 2
Structure / chemical formula		
Name	Fevipiprant	3,5-Bis(trifluoromethyl)phenol

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Figures

Figure 1:

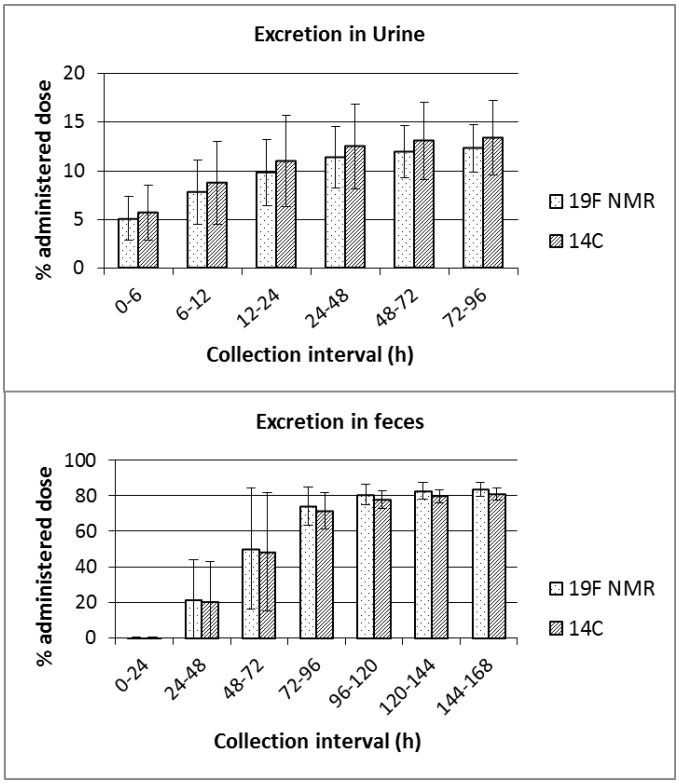

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Figure 2:

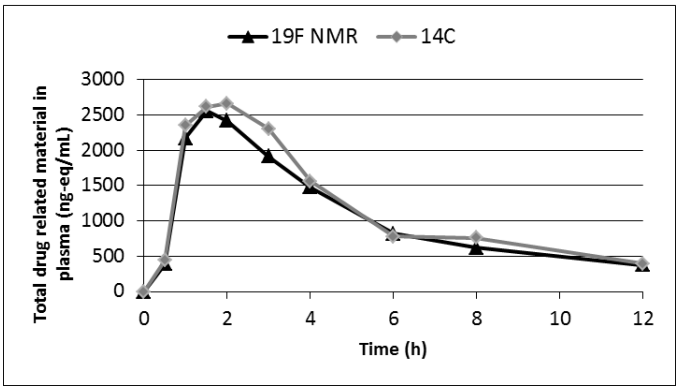


Figure 3

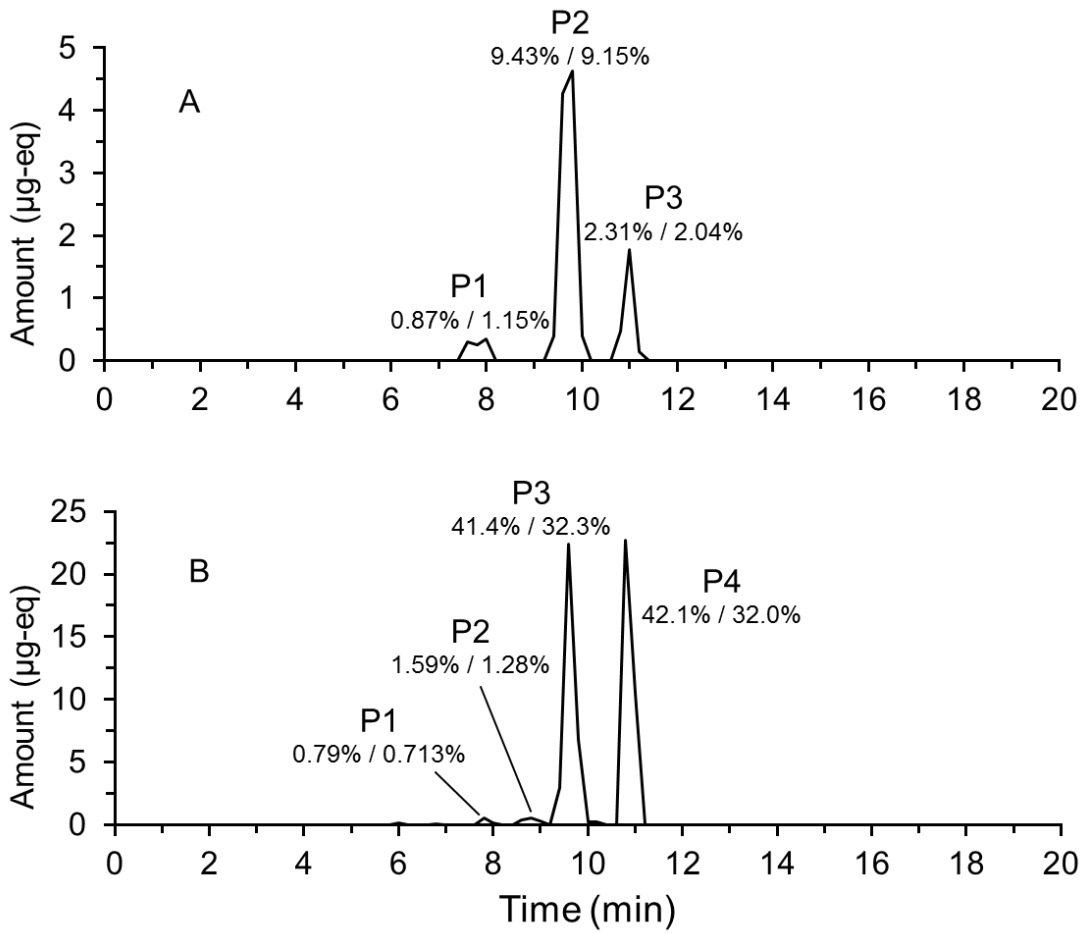


Figure 4

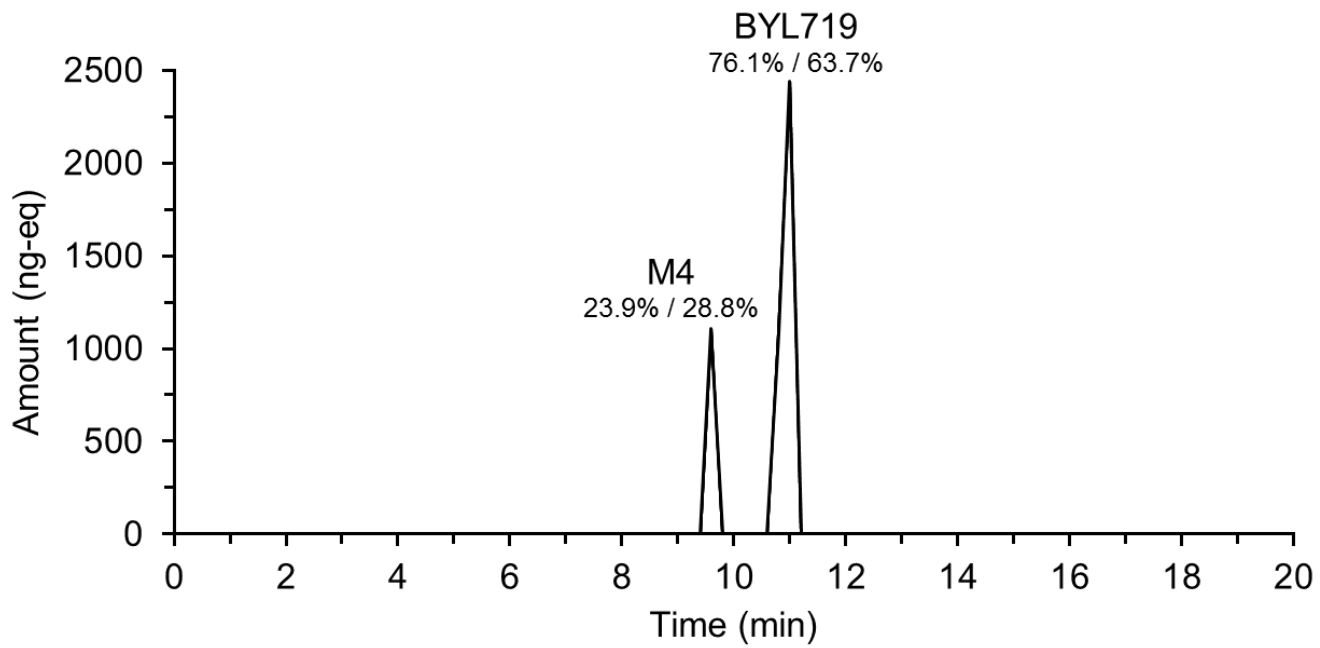
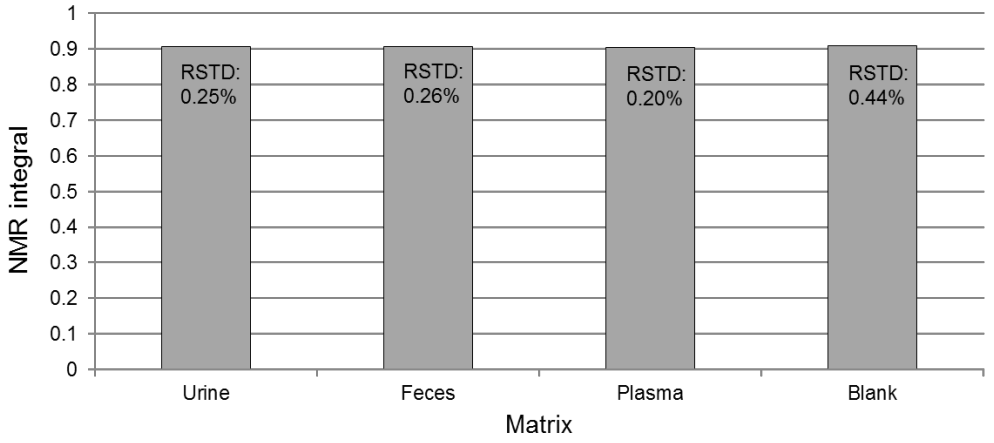


Figure 5



A

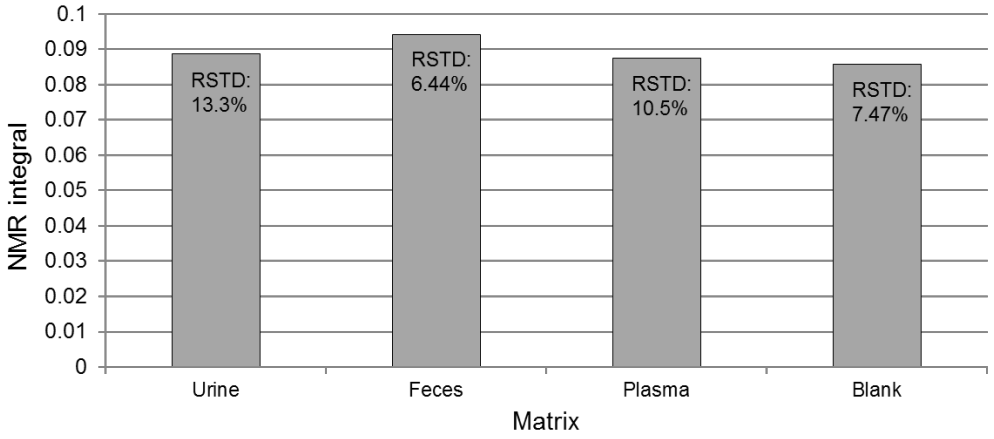


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Figure 6

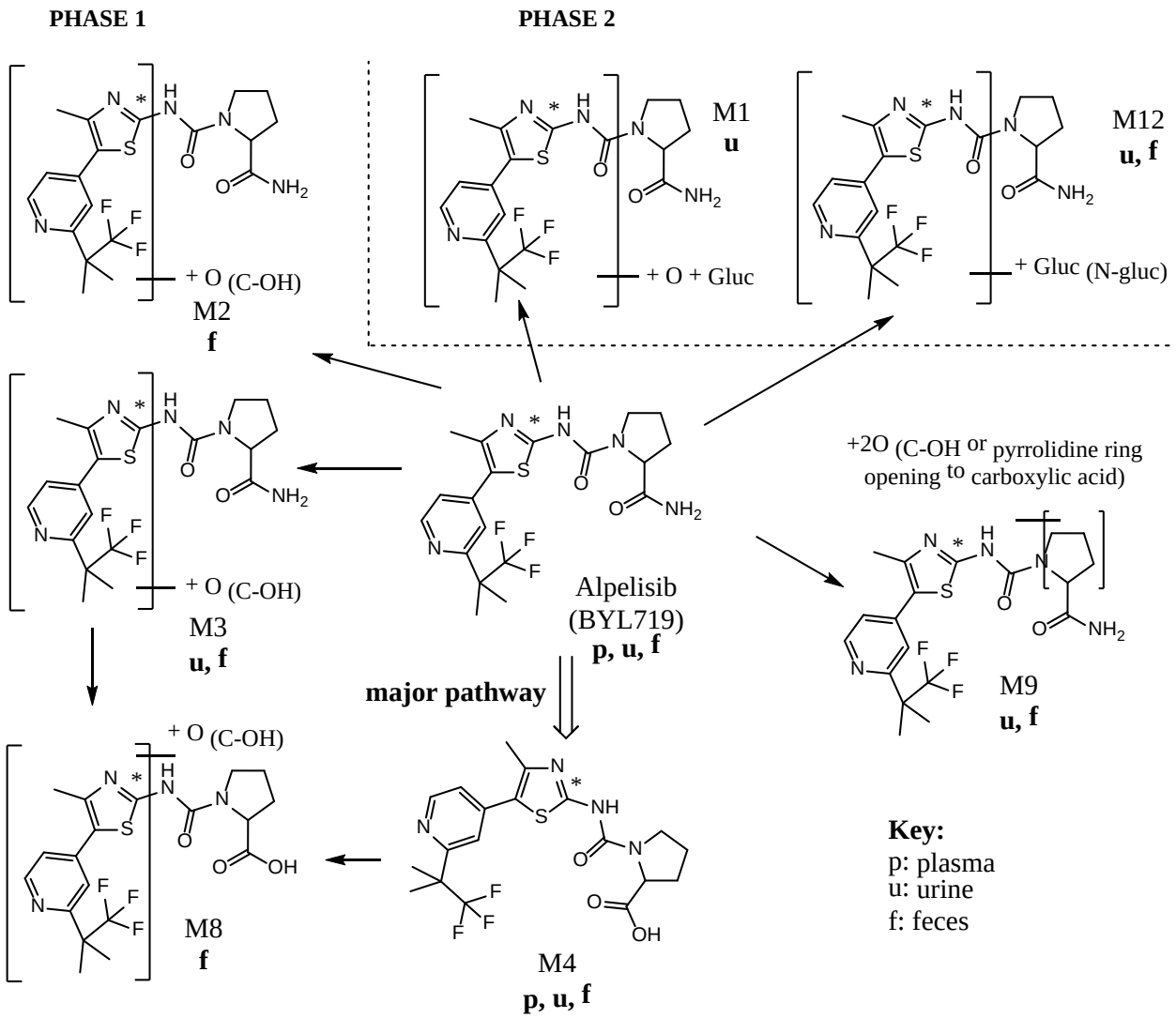

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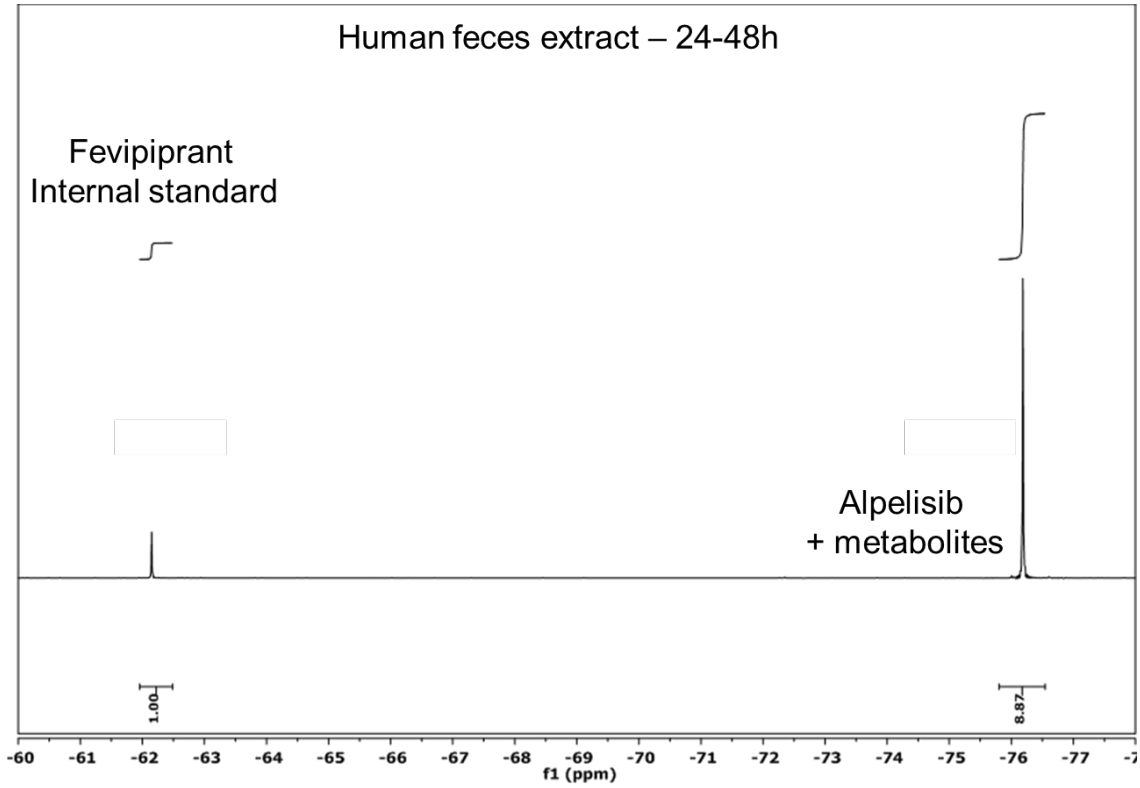
Figure 7



Comparison of ^{19}F-NMR and ^{14}C measurements for the assessment of ADME of BYL719 (Alpelisib) in humans.

James, Alexander David[1]; Marvalin, Cyrille[1]; Luneau, Alexandre[2]; Meissner, Axel[2], Camenisch, Gian[1].

Drug Metabolism and Disposition

SUPPLEMENTAL DATA

METHODS

Synthesis of [14C]BYL719

As a starting material to the synthesis of [14C]BYL719 acetamido-4-methylthiazole-2-14C was used (**2**) provided by ChemDepo. Heck coupling of 4-bromo-2-(1,1,1-trifluoro-2-methylpropan-2-yl)pyridine (**1**) with **2** in the presence of palladium acetate, tritertbutylphosphoniumtetrafluoroborate and cesium carbonate in dimethylformamide provided acetamide intermediate (**3**). The acetamide was deprotected using 2N solution of HCl in ethanol. The resulting amine (**4**) reacted with phenylchloroformate (**5**) in tetrahydrofurane in the presence of Hunig's base (N,N-diisopropylethylamine) afforded carbamates derivatives (**6** and **7**) which were coupled to L-prolinamide (**8**) afforded crude [14C]BYL719 (**9**). Last coupling was done in N,N-dimethylacetamide in the presence of catalytic amount of triethylamine. Purification of crude material by flash chromatography on silica gel followed by the suspension of evaporated fractions of interest in ethanol, filtration and drying of the filter cake provided very pure drug substance - [14C]BYL719 (**10**). Starting from 102.0 mCi of 2-acetamido-4-methylthiazole-2-14C, 31.1 mCi of (S)-N1-(4-methyl-5-(2-(1,1,1-trifluoro-2- methylpropan-2-yl)pyridin-4-yl)thiazol-2-^{14}C-2-yl)pyrrolidine-1,2-dicarboxamide was obtained. The overall yield of the synthesis was 30.5%.

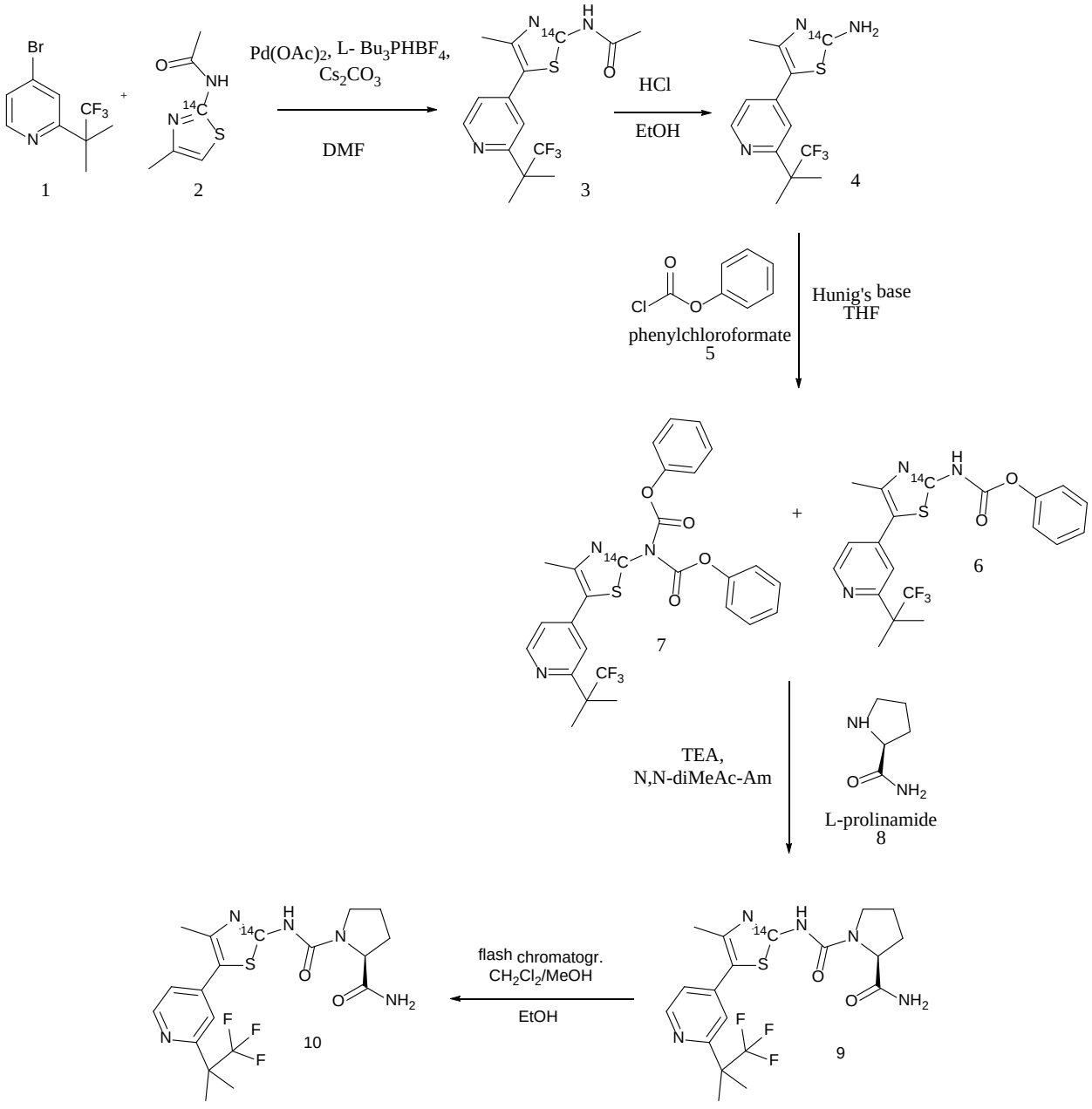


Figure S1. Synthetic scheme for [^{14}C]BYL719 (Alpelisib)

Synthesis of metabolite M4

The synthesis of metabolite M4 follows the same pathway as BYL719 (Furet et al, 2013) except for the last step which is replaced as follows (Figure S2):

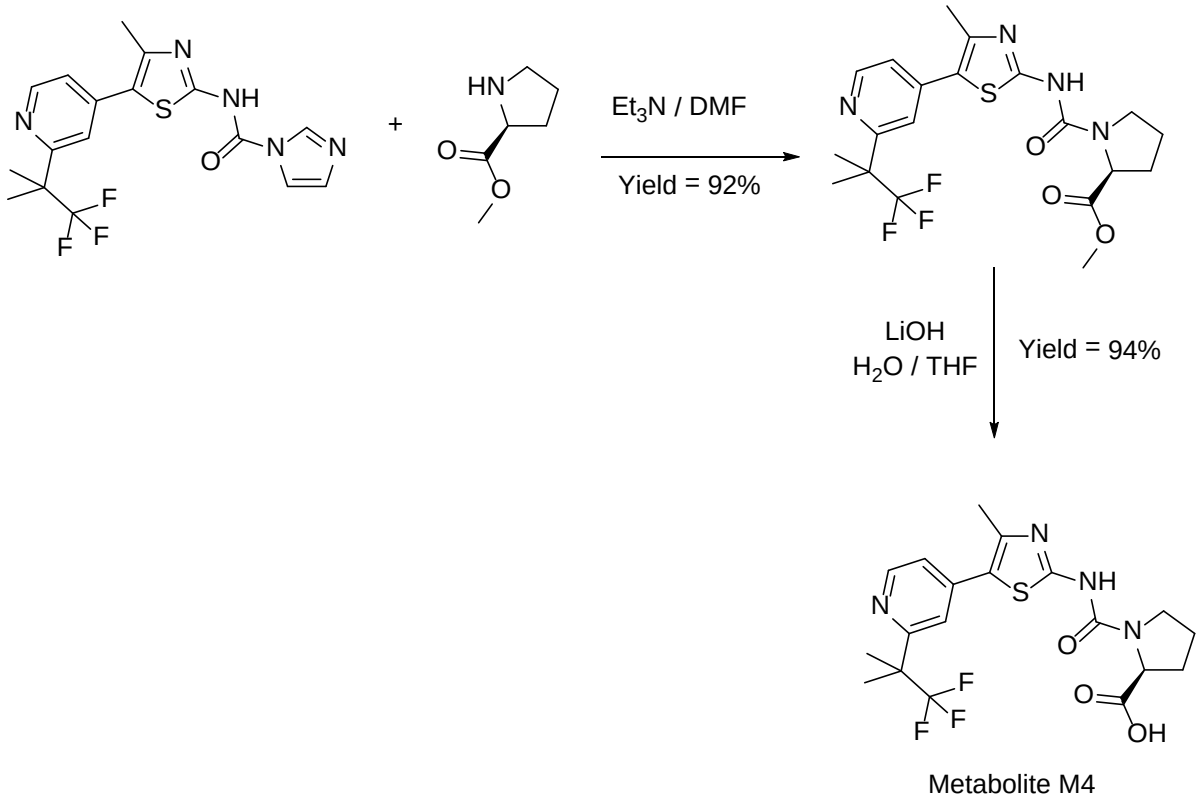


Figure S2. Synthetic scheme (last 2 steps) for metabolite M4. Preceding steps are published in Furet et al, 2013.

References

Furet P, Guagnano V, Fairhurst RA, Imbach-Weese P, Bruce I, Knapp M, Fritsch C, Blasco F, Blanz J, Aichholz R, Hamon J, Fabbro D, Caravatti G. (2013). Discovery of NVP-BYL719 a potent and selective phosphatidylinositol-3 kinase alpha inhibitor selected for clinical evaluation. Bioorg Med Chem Lett **23**: 3741-3748.