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Metabolites in Safety Testing: Issues and Approaches to the Safety Evaluation of Human Metabolites in a Drug that is Extensively Metabolized

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Abstract

In 2008 the Food and Drug Administration (FDA) modified its standard for evaluating toxicity of drug metabolites defining metabolites of concern as those that are detected circulating at more than 10% of the systemic exposure level of the parent compound at steady state. GSK1018921, a novel glycine transporter 1 inhibitor, was extensively metabolized in humans, with parent compound accounting for 12% and 1% of circulating drug related material after single and repeat dose, respectively. Since the parent was present at low relative concentrations, all fifteen metabolites detected in human plasma, met the 10% metabolites in safety testing (MIST) criterion, and therefore might require extensive quantification and further evaluation. At least thirteen metabolites warranted non-clinical characterization since they were either observed at significantly greater levels in humans than in preclinical species or they were not detected in animals. However the application of alternative strategies to 2008 FDA MIST guidance, such as those suggested by scientific literature and by the recent revision of the International Conference of Harmonisation (ICH) M3 guidance which recommended providing safety coverage for metabolites greater than 10% of total drug related material, allowed us to focus on one metabolite for additional safety evaluation.

Keywords: MIST guidance; ICH M3 guidance; Metabolite profiling; Human circulating metabolites; Safety evaluation; Metabolite quantification; GSK1018921; Induction

Abbreviations: ADME: Absorption Distribution Metabolism and Excretion; AUC: Area Under the plasma Concentration-time curve; C_{max}: Maximal plasma concentration; CYP: Cytochrome P450; FDA: Food and Drug Administration; HPLC: High Performance Liquid Chromatography; HPLC – MS: High Performance Liquid Chromatography – Mass Spectrometry; HQL : High Quantification Limit; ICH: International Conference of Harmonisation; LC-MS-MS: Liquid Chromatography tandem Mass Spectrometry; LLQ: Lower Limit of Quantification; MIST: Metabolite in Safety Testing; NMR : Nuclear Magnetic Resonance; NOAEL: No Observed Adverse Effect Level; ODRM: Observed Drug-Related Material; PXR: Pregnane-X-Receptor; QC: Quality Control; T_{max}: Time to reach C_{max}

Introduction

The issue of the role played by drug metabolites in the toxicity associated with their respective parent compounds has been a topic of growing interest to both the pharmaceutical industry and the regulatory agencies since publication of the MIST paper in 2002 [1]. Particular emphasis has been placed on those cases where metabolites circulating in humans are either absent in the animal species employed for toxicology testing or are present at much lower levels than in humans. In 2005, the U.S. FDA issued a draft guidance entitled "Safety Testing of Drug Metabolites" [2], in which the threshold for defining a major metabolite was 10% or more of the exposure to circulating drug-related material and in 2008 FDA released an updated guidance [3], which recommended providing safety coverage for metabolites representing greater than 10% of the systemic exposure of parent at steady state in humans. This was a significant challenge for the pharmaceutical industry. These challenges were even more problematic for cases where the parent circulated at very small quantities, cases where the metabolites had much longer half-lives than the parent and for compounds that were extensively metabolized and therefore showed multiple metabolites in human plasma at steady state which notionally require follow-up.

Multiple publications have addressed the lack of scientific consensus on the MIST issue [4-9] and several reviews and debates have been published on the issue of metabolite exposure threshold, necessitating additional non-clinical studies on the safety of metabolites and what these safety studies should be [1, 4-6, 10-13]. Additional non-clinical studies such as general toxicity, genotoxicity, embryo-foetal development, and carcinogenicity studies could imply the direct dosing of the major metabolite to the appropriate animal species. This approach would require synthesis of the major metabolite, testing in an appropriate preclinical species with associated toxicokinetic data "at least at levels comparable to those measured in humans" [3], and development of analytical methods capable of measuring the metabolite in non-clinical toxicology studies. There are costs and delays associated with this strategy that are not encountered when the metabolite is formed *in vivo*.

One of the major tasks in implementing the recommendation of FDA guidance was determining the exposure values of all metabolites

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that were found in human circulation in early development. Many researchers examine plasma and/or urine from the first human single and multiple dose escalation studies, in an attempt to obtain metabolic data earlier than the conduct of the radiolabeled study. Typically, human plasma samples from the highest dose of compound administered during the first multiple escalating dose study in healthy volunteers are used for the analysis and compared to animal plasma samples from multiple dose toxicology studies [14]. The ability to analyze human and animal plasma samples simultaneously greatly facilitates the comparison, and this may dictate the choice of toxicology study from which samples are used depending on the relative timing of the toxicology program and associated plasma sample availability. The metabolites discussed in this article were identified with a combination of high performance liquid chromatography and mass spectrometry (HPLC-MS). Semi-quantification of major metabolites in human plasma from single and escalating repeat dose studies was carried out using nuclear magnetic resonance spectroscopy (NMR) according to a methodology developed in house [15].

Since the work described in this article was performed in a timeframe between the release of 2008 FDA guidance and the recent revision of the ICH M3 guidance, this study provides an example when a compound, that is highly metabolized in humans and whose levels after repeated dose decrease significantly due to autoinduction, presents a particular challenge. In this case the majority of metabolites circulating in human plasma met the 2008 FDA guidance criteria of greater than 10% of parent and would, thus, theoretically have required further investigations. However the application of alternative strategies to MIST guidance, such as those suggested in the literature and in the recent revision to the ICH M3 guidance [16] allowed the selection of only one metabolite for which additional safety evaluation was recommended.

Materials and Methods

Chemicals and materials

HPLC grade acetonitrile and methanol were obtained from Fisher Scientific. Analytical grade ammonium acetate and glacial acetic acid were purchased from BDH. De-ionised water was generated in the laboratory using a Millipore Mill-Q water filter unit (Molsheim, France). Deuterium oxide was purchased from GOSS Scientific Ltd (Essex, UK). GSK1018921 (Figure 1) and its internal standard ($^{13}C_1{}^2H_3$-GSK1018921) was synthesised by GlaxoSmithKline Research and Development, Stevenage, UK.

Preparation and extraction of human plasma for metabolite identification and quantification

Human plasma samples were collected from healthy volunteers who received the highest dose [120 mg bis in die (BID)] orally administered in a repeat dose study to evaluate the safety, tolerability and pharmacokinetics of GSK1018921. Blank human plasma was provided by GlaxoSmithKline Spa, Verona, Italy. This study did not involve the use of radiolabelled drug.

Aliquots of plasma from 8 subjects were pooled (0-24 hours) in a time and volume dependent manner [17], to generate a sample representative of an area under the curve (AUC) exposure for parent and metabolites after Day 1 and Day 14. Aliquots of these 2 samples (60ml) were treated with 3 volumes of acetonitrile and vortex mixed (3min). The resulting precipitates were centrifuged (5000×*g*×30 min) and the supernatants were aspirated and dried down using heated nitrogen at 37°C. The residues were dissolved in methanol (5 ml) and reconstituted further by the addition of de-ionised water (45 ml). Further aliquots of the plasma pool and blank human plasma (1ml) were extracted as described above and reconstituted in 1ml of methanol:water (10:90 v/v) for use during LC-MS analysis.

Preparative HPLC

The entire sample extract (50ml) was separated by preparative HPLC with fraction collection using an Agilent HP1100 inject/collect autosampler coupled to a HP1100 binary pump and fraction collector (Agilent, Waldbronn, Germany). The eluent flow was split post column (100:1) between the fraction collector and a Waters Quattro Micro mass spectrometer (Waters, Milford, USA). Separations were carried out using a Phenomenex Luna 5µm Axia preparative HPLC column (100mm × 21.2mm i.d., Phenomenex, Torrance,CA, USA) at ambient temperature with a mobile phase composition of 10µM Ammonium acetate (pH adjusted to 5.0 with glacial acetic acid) and an acetonitrile gradient at a flow rate of 20ml/min. The proportion of acetonitrile was programmed at 18% at time 0 min, with an increase to 35% at 40min, and a final ramp to 95% at 40.1 min. HPLC eluent was collected into fractions, in a time-slice mode, into (6 ml) glass tubes at a rate of 15 s per fraction. This resulted in 160 fractions, each containing 5ml of column eluent. HPLC control was mediated through Chemstation (Agilent, Waldbronn, Germany) with the mass spectrometer controlled through Masslynx™ v.4.1 (Waters, Milford, USA). The fractions were taken to dryness under nitrogen at 37°C using a Micro DS96 dry down station (Porvair Scientific Ltd, Shepperton, UK) and then reconstituted in approximately 0.6 ml of deuterium oxide:acetonitrile (1:1) before being transferred to 5mm NMR tubes.

NMR analysis and quantification

NMR experiments were performed using a Bruker DRX-600 spectrometer equipped with an inverse 5mm TXI CryoProbeTM(1H/13C/15N) operating at 600.13MHz under the control of Topspin version 2.1 software (Bruker, Rheinstetten, Germany). ^{1}H NMR spectra were acquired using a standard NOESYPRESAT pulse sequence for solvent suppression with time shared double presaturation of the water and acetonitrile frequencies. In these experiments 128 transients were acquired into 64K data points over a spectral width of 12,019 Hz (20 ppm) with an inter-scan delay of 2.4 s giving a pulse repetition time of 5 s. ^{19}F NMR spectra were acquired without proton decoupling, for fluorine spectra 128 transients were acquired into 128K data points over a spectral width of 134,000 Hz (237 ppm) with an interscan delay of 3.0 s giving a pulse repetition time of 3.5 s. Routinely for proton data, the optimum receiver gain is determined solely by residual solvent signals due to the small amounts of material present in the isolated fractions, therefore, to improve inter-sample reproducibility an identical receiver gain was employed for all data acquisitions. Similarly for fluorine data due to the low absolute amounts of material present and the absence of endogenous fluorine an identical receiver gain was also used for all data acquisition. Prior to Fourier transformation, an exponential line broadening function of 1Hz was applied to each spectrum to improve the signal-to-noise ratio. Appropriate peaks were quantified using the integration features of Topspin version 2.1 software.

LC-MS analysis

Pooled human plasma extract and relevant NMR fractions containing drug related material were analysed qualitatively to assist metabolite identification using an Agilent HP1200 LC system (Agilent, Waldbronn, Germany) coupled to a ThermoFisher LTQ linear ion trap

mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA). Blank human plasma extract was also analysed as a control sample. Separations were carried out using a Thermo Beta Basic C18 5µm HPLC column (250mm × 4.6mm i.d. ThermoFisher Scientific, San Jose, CA, USA) at a temperature of 45°C with a mobile phase composition of 10µM Ammonium acetate (pH adjusted to 5.0 with glacial acetic acid) and an acetonitrile gradient at a flow rate of 1ml/min. The proportion of acetonitrile was programmed at 18% at time 0 min, with an increase to 58% at 60min, and a final ramp to 95% at 60.1 min. Flow was split post column (approximately 1:5 mass spectrometer: waste). HPLC control was mediated through Chemstation (Agilent, Waldbronn, Germany) with the mass spectrometer controlled through Xcalibur™ v.2.0 (ThermoFisher Scientific, San Jose, CA, USA)

Preparation and extraction of clinical and preclinical plasma samples for assessment of coverage of major human metabolites in preclinical species

Human plasma samples (0-24hr, day 14) were obtained from healthy volunteers who received 120 mg BID GSK1018921 orally during a repeat dose study. Plasma samples (0.5-24hr) from male and female rat dosed at the NOAEL dose of 20mg/kg/day during a 3 month toxicological study and plasma samples (0.25-24hr) from male and female dog dosed at the NOAEL dose of 6mg/kg/day during a 3 month toxicological study were used in this investigation. Female rabbit plasma samples (0.25-24hr) were obtained from a dose range finding study where the highest tolerated dose was 60mg/kg/day. Aliquots of plasma were pooled in a time and volume dependent manner [17], to generate a sample, for each species, representative of an area under the curve (AUC) exposure for parent and metabolites. Pooled control plasma for human and preclinical species was supplied by GlaxoSmithKline Spa, Verona, Italy.

Prior to extraction, aliquots of the human plasma pool were mixed with an equal volume of control plasma from each of the preclinical species. Similarly, for each of the preclinical species samples, an equal volume of human control plasma was added to ensure all samples contained similar co-extractives prior to LC/MS/MS analysis. Aliquots of all samples (600µl) were treated with 3 volumes of acetonitrile containing internal standard $^{13}C_1{}^{2}H_3$-GSK1018921 (7.5ng/ml) and vortex mixed (3min). The resulting precipitates were centrifuged (1200×*g*×10 min). The supernatants were removed, the pellets were resuspended in acetonitrile (1.8ml) and the process repeated. Supernatants were combined and dried down using heated nitrogen at 37°C. Finally, the residues were reconstituted in 300µl of water/acetonitrile (70:30).

LC/MS/MS analysis for assessment of coverage of major metabolites in preclinical species

All semi-quantitative LC/MS/MS experiments were conducted using an Agilent 1100 HPLC system (Waldbronn, Germany) with a CTC HTS Pal autosampler (CTC Analytics, Zwingen, Switzerland) coupled to an API- 4000 triple quadrupole mass spectrometer controlled through Analyst™ and equipped with a TurboIonspray® (TISP) source (Applied Biosystems, Toronto, Canada). Separations were carried out as previously described. Analytes and a stable labelled internal standard of the parent drug were detected by tandem mass spectrometry (MS/MS), using selected reaction monitoring (SRM) of appropriate transitions. Chromatographic peaks were integrated using Analyst™ (Applied Biosystems, Toronto, Canada). Analyte/internal standard MS peak area ratios for each metabolite in human plasma was compared with the analyte/internal standard MS peak area ratio of the same metabolite in rat, dog and rabbit plasma [18]. The ratio between these is described in the following equation:

Ratio = C/D

C= MS response (analyte/internal standard peak area ratio) of circulating human metabolite detected in preclinical species plasma

D= MS response (analyte/internal standard peak area ratio) of circulating human metabolite detected in human plasma.

Data analysis

The estimates of metabolite exposure both in terms of theoretical maximum concentration (C_{max}) and in terms of area under the plasma concentration-time curve (AUC), were calculated from parent clinical C_{max} and AUC_{0-24} as detailed in the following formula:

$$C_{max}\ \text{metabolite} = \frac{\text{Clinical } C_{max}\ \text{(parent)} * \%\ \text{ODRM (metabolite)}}{\%\ \text{ODRM (parent)}}$$

$$\text{AUC metabolite} = \frac{\text{Clinical AUC (parent)} * \%\ \text{ODRM (metabolite)}}{\%\ \text{ODRM (parent)}}$$

ODRM: Observed drug-related material (Generated by ^{19}F NMR quantification)

In vivo testing of metabolite SB636406

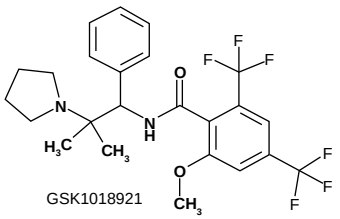
In general toxicology studies, SB636406 was given by oral gavage to rats at 30 or 150 mg/kg/day (males) or at 15 or 50 mg/kg/day (females) once daily for 6 weeks and to male and female dogs at doses of 20, 50 or 100 mg/kg/day for 7 days. In embryo-foetal development toxicity studies, SB636406 was given orally at 20 or 60 mg/kg/day to pregnant female rabbits on Days 7 through 19 pc. Three animals/sex/group (rat and dog) or nine animals/group (rabbit) were used at each dose level for toxicokinetic evaluation. The research described in this paper complied with national legislation and with the company policy on the care and use of laboratory animals and with related codes of practice.

Sampling

Plasma samples obtained from blood collected into tubes containing EDTA, by mixing gently, placing on crushed wet ice and centrifuging as soon as possible, were collected on Day 1 and Day 43 (rat), on Day 1 and Day 7 (dog) and on Day 5 (Day 11 postcoitum; rabbit) at the following nominal times: predose (Day 43 and Day 7 only in rat and dog, respectively), 0.5, 1, 2, 4, 8 and 24hours after dosing and were stored at nominally -20°C until analysed as previously described.

Quantification of metabolite SB636406

Plasma samples were analysed for SB636406 (Figure 1) by using a validated analytical method based on protein precipitation with acetonitrile containing stable labelled internal standard ($^{13}C^{2}H_3$-SB-636406). Samples were analysed using a Waters Acquity (Waters, Milford, USA) coupled to an Applied Biosystems/MDS Sciex API-5000 mass spectrometer controlled through Analyst™ and equipped with a TurboIonspray® (TISP) source (Applied Biosystems, Toronto, Canada). Separations were carried out using a Phenomenex Luna PFP (50mm × 2.1mm i.d. 3µm) at a temperature of 40°C with a mobile phase composition of water and an acetonitrile gradient at a flow rate of 0.8mL/min. The proportion of acetonitrile was programmed at 20% at time 0 min, held isocratically for 0.2min, followed by a ramp



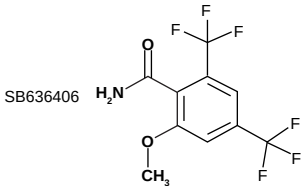


Figure 1: Structures of GSK1018921and its metabolite SB636406.

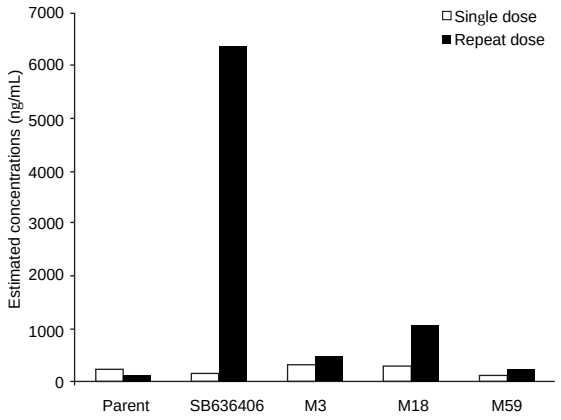


Figure 2: Concentration Estimates of Notable Metabolites Following Single and Repeat Oral Dosing of GSK1018921 in healthy volunteers.

to 80% at 0.8min and finally a further isocratic period of 0.1min. Flow was not split post column. The lower limit of quantification (LLQ) for SB636406 was 100ng/ml (rat and rabbit) or 20ng/ml (dog), using a 25µl aliquot of rabbit, rat or dog plasma with a higher limit of quantification (HQL) of 100000 ng/ml (rat and rabbit) or 20000ng/ml (dog). The computer systems that were used to acquire and quantify data included Analyst™Version 1.4.2 and SMS2000 Version 2.1.

Quality Control samples (QC), prepared at 3 different analyte concentrations and stored with study samples, were analysed with each batch of samples against separately prepared calibration standards. QC samples and calibration standards were prepared using independently prepared stock solutions of SB636406 reference material. For the analysis to be acceptable, no more than one-third of the QC results were to deviate from the nominal concentration by more than 15% and at least 50% of the results from each QC concentration were to be within 15% of nominal. The applicable analytical runs met all predefined acceptance criteria.

Toxicokinetic analysis

Toxicokinetic analysis was performed by noncompartmental pharmacokinetic analysis using WinNonlin™, Enterprise Edition Version 4.1. All computations utilised the nominal sampling times.

The systemic exposure to SB636406 was determined by calculating the area under the plasma concentration-time curve (AUC) from the start of dosing to the last quantifiable time point (AUC_{0-t}) using the linear-logarithmic trapezoidal rule. In rabbit, the mean concentration at 24 hours was used as the zero hour value in calculating the portion of the AUC between the start of dosing and first sampling occasion. The maximum observed peak plasma concentration (C_{max}) and the time at which it was observed (T_{max}) were determined by inspection of the observed data. The numerical data presented in the table are computer generated; because of rounding, recalculation of derived values from individual data presented in the table will, in some instances, yield minor variation.

Results

The metabolic profile of GSK1018921 has been determined in plasma of healthy volunteers following single and 14-day repeat oral administration of parent compound. In order to maximize the amount of drug-related material and thereby facilitate metabolite identification, the analysis involved samples generated following the highest dose of compound [240 mg (120 mg BID)] orally administered to humans. In order to assess the coverage of major human metabolites in preclinical species, human plasma samples from the last day of dosing from the multiple dose study in healthy volunteers were compared to animal plasma samples from multiple dose toxicology studies.

Human metabolite identification and quantification after single dose

Human circulating metabolites were identified by a combination of LC-MS analysis, followed by preparative HPLC and ^{1}H NMR of fractions (data not reported). Identified metabolites were subsequently quantified by reanalysing fractions by ^{19}F NMR, where data for each metabolite was expressed as percentage of observed drug related material (ODRM). ^{19}F NMR can clearly only be used in cases such as this where the molecule contains Fluorine atoms. In other cases, the quantification can often be carried out using ^{1}H NMR of a suitable aromatic proton [15]. Following this analysis, the circulating components identified in humans were parent compound, accounting for 12% of the observed circulating drug-related material (DRM), a product of N-dealkylation resulting in cleavage of the molecule (SB636406; 8% DRM), a N, N debutylated derivative (M3; 17% DRM), an oxidised derivative (M18; 16% DRM) and a N, N debutylated, hydroxylated O-glucuronide derivative (M59; 6% DRM) (Table 1). Another minor metabolite, a N, N-debutylated and hydroxylated derivative (M24), representing less than 1% of drug related material was also detected. The authors recognised that some of the metabolites were generated from the right hand side of the molecule, in particular SB636406. This leaved the possibility that a sizeable fragment from the left hand side of the molecule could exist as a metabolite and would not be detected by the ^{19}F quantitative methodology used here. All fractions generated by the preparative HPLC method were also screened by ^{1}H NMR to attempt to detect this additional fragment / metabolite. However no evidence for this was found.

Human metabolite identification and quantification after repeat dose

Parent compound and all the metabolites described above, with the exception of M24, were detected in human plasma collected from healthy volunteers after 14 days of oral administration of 240 mg (120 mg BID) GSK1018921. Parent compound accounted for 1% of the circulating drug-related material and the metabolites SB636406, M3,

Peak ID	Single dose %ODRM[a] (% of parent)	Repeat dose %ODRM[a] (% of parent)	Exposure multiple[b] rat vs human	Exposure multiple[b] dog vs human	Exposure multiple[b] rabbit vs human
Parent	12	1	M = 13 F = 10	M = 17 F = 15	1
SB636406 N-dealkylation	8 (67)	54 (5400)	M = 0.1 F = 0.1	M = 0.1 F = 0.1	0.2
M3 N,N-debutylation	17 (142)	4 (400)	M = 1 F = 1	M = 2 F = 1	1
M18 Oxidation	16 (133)	9 (900)	M = 4 F = 9	M = 0.7 F = 0.4	0.3
M59 N,N-debutylation, hydroxylation and O-glucuronidation	6 (50)	2 (200)	NA	M = 0.05 F = 0.03	0.1

For exposure estimates rounding of data was carried out at the final stage for presentation purpose only and minor numerical differences may be observed.
ODRM = Observed Drug Related Material
[a]Determined by ^{19}F NMR integration.
[b]0-24 hours proportional pool after repeat dosing in rat, dog, rabbit (at the NOAEL) and human (at 120 mg BID)
NA = Not Applicable. M59 was not detected in rat plasma.
M = Male
F = Female

Table 1: Notable Human Metabolites Identified in Pooled Plasma Extracts and Their Exposure Ratio Estimates in Pooled Plasma of Preclinical Species.

Peak ID	Single dose %ODRM[a] (% of parent)	Repeat dose %ODRM[a] (% of parent)
Parent	12	1
M19 Oxidation	ND	<3 (<300)
M24 N,N-debutylation and hydroxyation	<1 (<8)	ND
M27 N,N-debutylation, O-demethylation, O-glucuronidation	ND	<1 (<100)
M28 N-dealkylation, O-demethylation, O-sulfation	ND	<1 (<100)
M65 Dehydrogenation and hydroxylation	ND	<3 (<300)
M75 N,N-debutylation, oxidation to acid, di-hydroxylation and O-glucuronidation	ND	2 (200)
M76 O-demethylated M59	ND	<1 (<100)
M77 N,N-debutylation, hydroxylation, oxidation to acid and glucuronidation	ND	1 (100)
M78 O-demethylated M77	ND	2 (200)
M79 Oxidation	ND	<1 (<100)
M80 Amide hydrolysis, O-demethylation	ND	1 (100)
M81 N-dealkylation and hydroxylation	ND	<1 (<100)

[a]Determined by ^{19}F NMR integration.
ODRM = Observed Drug Related Material.
ND = Not Detected.

Table 2: Additional Minor Human Metabolites Identified in Pooled Plasma Extracts Following Single and Repeat Oral Administration of GSK1018921 to Healthy Volunteers.

M18 and M59 accounted for 54%, 4%, 9% and 2% of circulating drug related material, respectively (Table 1). Eleven additional metabolites, each representing 3% or less of circulating drug-related material were also detected (Table 2). It should be noted that the majority (55%) of these metabolites were conjugates of metabolites formed by N-N debutylation and hydroxylation. Several minor metabolites were also detected by MS only. Since parent compound represented a negligible percentage of circulating drug-related material, all the

Peak ID	Mean C_{max} (ng/mL)	Mean C_{max} (μM)	% SB636406
Parent	118[a]	0.2	-
SB636406 N-dealkylation	6372	22.1	-
M3 N,N-debutylation	472	1.1	7.4%
M18 Oxidation	1062	2.1	17%
M19 Oxidation	<354	<0.7	<5.6%
M27 N,N-debutylation, O-demethylation, O-glucuronidation	<118	<0.2	<1.9%
M28 N-dealkylation, O-demethylation, O-sulfation	<118	<0.3	<1.9%
M59 N,N-debutylation, hydroxylation and O-glucuronidation	236	0.4	3.7%
M65 Dehydrogenation and hydroxylation	<354	<0.7	<5.6%
M75 N,N-debutylation, oxidation to acid, di-hydroxylation and O-glucuronidation	236	0.4	3.7%
M76 O-demethylated M59	<118	<0.2	<1.9%
M77 N,N-debutylation, hydroxylation, oxidation to acid and glucuronidation	118	0.2	1.9%
M78 O-demethylated M77	236	0.4	3.7%
M79 Oxidation	<118	<0.2	<1.9%
M80 Amide hydrolysis, O-demethylation	118	0.4	1.9%
M81 N-dealkylation and hydroxylation	<118	<0.4	<1.9%

[a]Clinical C_{max}

Table 3: Mean Maximum Concentration Estimates of Metabolites Identified in Pooled Human Plasma Extracts at Steady State and Their Percentages Relative to Metabolite SB636406.

metabolites identified in human plasma circulated at levels above 10% of the parent area under the curve (AUC) at steady state resulting in a total of fifteen metabolites exceeding the 2008 FDA criterion. After multiple dosing the parent concentration decreased 2-fold (*data not reported*) and, based on the concentration estimates, the levels of metabolites SB636406, M18 and M59 increased 42 -, 3.5 - and 2 - fold, respectively with metabolite SB636406 exceeding the levels of parent by approximately 54-fold, whilst the levels of metabolite M3 did not change (Table 3 and Figure 2). Furthermore metabolites SB636406, M3 and M18 after repeat dose administration of parent compound reached estimated circulating concentrations equal or greater than 1μM (Table 3).

Assessment of coverage of major human metabolites in preclinical species

Following comparison of repeat dose human plasma samples from the last day of dosing with rat, dog and rabbit plasma samples from multiple dose toxicology studies at the NOAEL, using the semiquantitative LC-MS-MS approach described above, metabolite SB636406 was present in disproportionately lower levels in toxicology species whilst metabolites M3 and M18 were present in equal or greater quantities in all the preclinical species used in toxicological assessment. The phase II metabolite M59 was not detected in rat plasma and was present in disproportionately lower levels in dog and rabbit (Table 1).

Regarding the eleven metabolites detected after multiple dose and representing 3% or less of drug-related material, one (M19) was a minor circulating entity in rat and only two (M27 and M28) were detected in excreta (bile or urine) in at least one preclinical species, thereby indicating that the animals have been exposed to these metabolites.

In vivo testing of metabolite SB636406

In the repeat dose toxicology studies, different doses of SB636406 were orally administered to preclinical species used in toxicological assessment (rat, rabbit and dog) and the toxicokinetics of SB636406 were investigated by use of a validated LC-MS-MS assay to ensure that levels comparable to those estimated in humans were achieved.

Following oral administration of SB636406 once daily for 7 days in dog, for 6 weeks in rat and on Day 7 to Day 19 postcoitum in mated female rabbits, the no observed adverse effect level (NOAEL) in male

Species (Duration)	Dose (mg/kg/day)	Sex	C_{max} (µg/mL)		AUC_{0-24} (µg.h/mL)	
			Day 1[a]	End of Study	Day 1[a]	End of Study
Rat (6 weeks)	15	F	5.37	4.06	103	45.9
	30	M	10.5	4.45	121	28.7
	50	**F**	**22.1**	**15.9**	**424**	**221**
	150	**M**	**36.3**	**15.3**	**465**	**120**
Dog (7 Days)	20	M	5.32	4.72	42.2	39.2
		F	14.8	6.12	71.8	31.7
	50	M	21.1	7.80	151	46.8
		F	25.7	7.22	236	41.1
	100	M	15.2	11.0	184	62.5
		F	15.0	13.4	95.5	36.4
Rabbit (EDF)	**20**	**F**	**8.03**	**-**	**73.1**	-
	60	F	24.6	-	246	-
Human (repeat dose)	240 mg[b] (120mg BID)	M	0.149[c]	6.37[c]	1.75[c]	68.5[c]

Note: End of study was Day 43 for rats, Day 7 for dogs and Day 14 for humans

The no adverse effect levels (NOAELs) are in **bold type**

EDF = Embryofetal development

[a]Day 5 of dosing (Day 11 postcoitum) for rabbit

[b]GSK1018921 administered in the repeat dose study

[c] Estimates of metabolite exposure in terms of theoretical C_{max} or AUC_{0-24} were calculated from clinical C_{max} and AUC_{0-24} data of parent considering % of observed drug related material

Table 4: Mean Systemic Exposure to SB636406 Following Repeat Oral Administration of SB636406 to Rats, Dogs and Pregnant Rabbits.

and female rats were 150 mg/kg/day and 50 mg/kg/day, respectively and in female rabbits was 20 mg/kg/day, while 100 mg/kg/day was the highest dose tested in dog (*data not reported*).

Systemic exposure (AUC_{0-t} and C_{max} values) to SB636406 generally increased approximately proportionally with increasing dose in rat, dog and rabbit and decreased up to 4-fold in rat and up to 6-fold in dog following repeat administration of SB636406 (Table 4). Systemic exposure to SB636406 showed a trend towards higher exposure in female rats when compared to male rats.

The systemic exposures (AUC_{0-t} and C_{max} values) to SB636406 achieved in rat and rabbit after repeat dosing of SB636406, at the NOAEL, were greater than those estimated in human following repeat administration of 120 mg BID GSK1018921, indicating that both preclinical species were adequately exposed to SB636406. On the contrary in dog systemic exposures to SB636406 decreased after 7-day repeat oral administration to levels that were still lower than those estimated in human (Table 4).

Discussion

In this article we have provided an example of the potential impact of the 2008 FDA MIST guidance both on the number and nature of metabolites requiring additional quantification and safety evaluation and on drug development workload and timelines.

Investigation of the human metabolic profile of GSK1018921 in plasma samples from the first repeated dose study in healthy volunteers revealed that GSK1018921 was extensively metabolized, since parent compound represented only 12% and 1% of circulating drug related material after single and after multiple oral dose, respectively.

The decrease in systemic exposure to parent compound observed after repeat dose was likely due to induction by GSK1018921 of its own metabolism. As a consequence of auto-induction, the number of metabolites and the estimated levels of metabolites detected after repeat dose increased significantly with respect to single dose. After multiple administration, the parent drug comprised 1% of circulating drug-related material and there was just one metabolite (SB636406) in circulation that was present at >10% of circulating drug-related material. However, there were 14 more metabolites present at between 1 and 10% of circulating drug-related material, which placed them at >10% of parent drug and hence potentially warranting further consideration. Among these 14 metabolites two (M3 and M18) were found to have sufficient coverage in toxicological species, two (M19 and M59) were minor circulating entities in animal species, two (M27 and M28) were detected only in animal excreta (bile and/or urine) and eight metabolites, each representing 3% or less of circulating drug-related material were not observed in animals. Classification of the last 8 metabolites indicated that half (M75, M76, M77 and M78) were conjugates of metabolites formed by N,N-debutylation and oxidation, two metabolites (M65 and M79) were formed by oxidation and the remaining metabolites were formed by amide hydrolysis and O-demethylation, (M80) and by hydroxylation (M81) of SB636406. If the analysis on human metabolites was performed following the criteria described in the FDA's initial draft guidance for Industry: Safety Testing of Drug Metabolites (2005), where major metabolites were defined as those metabolites above 10% of drug-related circulating material, only SB636406 would meet the threshold. However, in view of the 2008 FDA MIST guidance, the only one available at the time the work described here was performed, all the metabolites detected in human plasma were considered major since the parent itself was present at a low relative percentage. Therefore at least six metabolites (thirteen including phase II metabolites) potentially needed further investigation, even those accounting for only 1% of drug related material and this strategy if implemented would have had a significant impact on costs, workload and delay in the timelines for drug development.

A similar situation has been described by Smith and Obach [19] who reported 15 examples from the recent scientific literature of human radiolabeled ADME studies published from 2006 onward. If the criteria for detailed consideration were based solely on 10% of total drug related material, then theoretically, a maximum of 10 metabolites could meet the threshold and be considered major metabolites. However, if based on 10% of the parent, the number of

major metabolites could be almost infinite if the parent itself was present at a low relative percentage. In their analysis, four of the 15 compounds had one or two major metabolites that were not detected in animal circulation, 5 of 14 compounds had four or fewer major metabolites per compound and in one case, there were 13 major metabolites that were minor in animals.

In our case the issue around the definition of major metabolites was also addressed following a recent revision to the overall ICH guidance on non-clinical safety assessment [16] which recommends that non-clinical characterization of a human metabolite(s) is only warranted when that metabolite(s) is observed at exposure greater than 10% of total drug-related exposure and at significantly greater levels in humans than the maximum exposure seen in the toxicology studies. In our case, only metabolite SB636406 met the criteria of the ICH M3 guidance and therefore needed further safety characterization. From a regulatory perspective, the 2009 revisions to the ICH M3 guidance take precedence over prior regional regulatory guidance on this issue. Although additional regulatory guidance on MIST will likely be provided in the future, the use of the ICH criteria for the selection of circulating human metabolites that may require separate safety assessment studies appears to be appropriate.

Another approach applied in our case was to consider absolute estimated concentrations of circulating metabolites. According to Smith and Obach [6], for human metabolites that are structurally distinct from the parent drug but are present in circulation at high concentrations (e.g., > 1μM), additional safety evaluation for an appropriate risk assessment may be required, especially if human exposure is greater than that observed in animals. In our case, metabolites SB636406, M3 and M18 were present in human plasma at estimated concentrations higher than 1μM. However, metabolites M3 and M18 had sufficient coverage in preclinical species, while SB636406, that was structurally distinct from the parent and present at an estimated concentration of 22μM, was circulating at disproportionately higher levels in human than in any of the toxicological species and therefore needed additional safety evaluation.

The last criteria applied in our case for definition of a major metabolite was the one suggested in a recent public scientific ISSX meeting [20] held in San Diego (USA). This considers that, when the parent is low or absent, major metabolites are those peaks found to represent >10% of the major peak (in these cases a metabolite). In our case the major peak was represented by SB636406, accounting for 54% of circulating drug-related material and the major metabolite was considered M18, being 17% of SB636406. However, since M18 was shown to circulate in animals at levels equal or greater than those seen in human, no further safety testing of this metabolite was evaluated.

The FDA metabolite guidance supported and encouraged a scientific, rational and flexible approach as well as the case-by-case assessment in decision making. Considering this, we have implemented

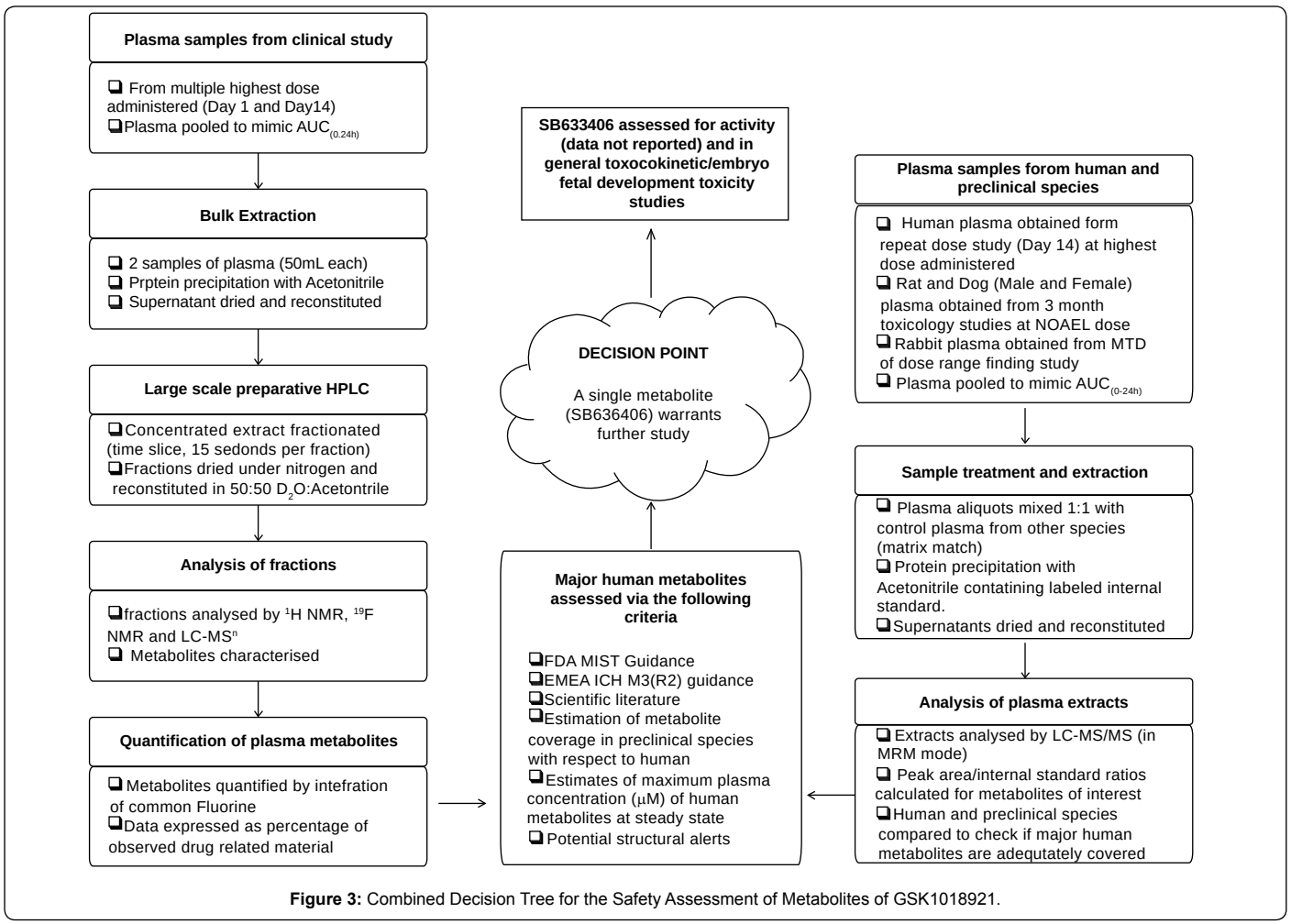


Figure 3: Combined Decision Tree for the Safety Assessment of Metabolites of GSK1018921.

a strategy based on all the criteria described above to define which major human metabolites of our compound warrant further testing in non clinical species (Figure 3). Since the contribution of SB636406 to clinical risk was unknown because its potential toxicity in animals was not adequately investigated, this metabolite needed further evaluation in non-clinical studies to determine its toxicological potential. Therefore it was decided to synthesize large quantities of SB636406 for further safety testing and to dose SB636406 directly to the appropriate animal species. The non-clinical studies performed to qualify metabolite SB636406 included general toxicology studies in rat and dog, the preclinical species used to test safety of the parent compound and embryo-foetal development studies in rat and rabbit. After a preliminary 7-day toxicology study in dog, this species was excluded from further safety testing of metabolite since systemic exposures to SB636406 decreased after repeated oral administration and at the end of the study were still lower than those estimated in human. On the contrary, in rat systemic exposure to SB636406 following 7-day oral administration of SB636406 was higher than that achieved in dog and similar to that estimated in human, therefore rat was selected as the best species to test potential toxicity of the metabolite for a maximum of 6 weeks in order to support any clinical development trial up to 6 weeks duration. The decision taken was in agreement with the context of the MIST Guidance [3], which states that coverage for disproportionate metabolites is considered sufficient if the metabolite circulates in at least one animal species at concentrations approaching those found in human circulation at pharmacologically relevant doses and in at least one of the rodent species that are used for 2 year carcinogenicity testing.

In both rat and rabbit systemic exposures to SB636406 after repeated dosing, at the NOAEL, were similar to or greater than those estimated in human following repeated administration of 120 mg BID of GSK1018921, indicating that both preclinical species were adequately exposed to SB636406.

In conclusion, we have reported a scenario where the presence of low levels of parent compound, as a consequence of extensive metabolism, had posed some challenges in assessing all metabolites that were present in greater than 10% of parent AUC according to the recommendation of the 2008 FDA guidance that was released when the study here described was performed. The recent revision of the ICH M3 guidance had a strong and positive impact on our case since we have used the ICH criteria for the selection of circulating human metabolites that had required separate safety assessment studies.

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