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Evaluation of preparative high performance liquid chromatography and cryoprobe-nuclear magnetic resonance spectroscopy for the early quantitative estimation of drug metabolites in human plasma

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ABSTRACT

Definitive information on the metabolism of a drug candidate in humans is achieved through dosing radiolabelled drug as part of a clinical study, and is typically conducted post-proof of concept in Phase III of the clinical development plan. Here we describe a novel approach, using preparative high performance liquid chromatography and cryoprobe-nuclear magnetic resonance spectroscopy, to determine the human systemic exposure to a drug and its metabolites using samples derived from Phase I clinical studies. Using the described methodology, novel human plasma metabolites, as low as 10 ng/ml can be detected and quantified. This provides an opportunity, early in the development process to understand the potential role of metabolites in the safety and efficacy of drugs in humans.

1. Introduction

Having an appreciation of the human metabolism of putative drugs early in the development process can have important consequences for the assessment of a compound's safety and efficacy. Many drugs on the market have been shown to have active metabolites that can have a better efficacy profile than the original parent compound [1]. Other drugs are known to have toxic metabolites that may not have been adequately evaluated prior to administration in a large population [2,3]. Traditionally, an initial understanding of human metabolism has relied on *in vitro* metabolism from isolated human hepatocyte or microsomal preparations and the *in vivo* metabolism from a human radiolabelled study (HRS) [4]. However, due to ethical implications and complexity of the study design, human radiolabelled studies are often conducted during Phase III of the drug development process and less frequently during the earlier phases. This means that the opportunity for monitoring metabolites contributing to safety and efficacy in Phase II studies may have been missed. If human metabolites are unique or poorly represented in preclinical species, this could lead to a halt in the clinical investigations while these components are evaluated for toxicological liabilities. Having an earlier idea of the metabolism in humans would mean that consideration of any safety aspects could be made prior to exposing larger numbers of patients or subjects to such risks that may be associated with new chemical entities.

Earlier information on human metabolism can be obtained by collecting urine from subjects on Phase I studies and examining this for metabolites using either mass spectrometry (MS) [5] or nuclear magnetic resonance (NMR) spectroscopy [6–9]. Analysis of urine, by NMR, has the advantage of providing more definitive information on the structures of metabolites and provides a reliable estimate of the relative proportion of each metabolite because it is an inherently quantitative technique [10]. However, the importance of urine as a route of elimination in humans is unlikely to be known at this stage of development and as such the metabolites identified may be misleading, when considering the significance of an individual metabolite to the overall disposition and safety of a drug in humans.

At present, a common surrogate for the assessment of a drug's safety is to measure systemic exposure of the drug in toxicology species, compare this to the exposure seen in human, and then set safety limits based on these data. More recently there has been much interest in including metabolites in such considerations [11–14]. The recently published Food and Drug Administration (FDA) Guidance for Industry on Safety Testing of Drug Metabolites (FDA, 2008), highlights the need to adequately assess the safety of unique and/or major human metabolites [15], implying some assessment of circulating metabolite quantities is required. However, the levels of metabolites in human plasma are relatively

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low, and detection and characterisation has almost exclusively relied on the use of MS [16]. Although, MS can provide many structural clues as to a metabolite's identity, it is generally not definitive and without reference standards, the determination of the amount of each metabolite is often unreliable due to differences in ionisation efficiency and the influence of the underlying matrix [17].

The recent development of cryoprobe technology for NMR [18,19] has improved the sensitivity of this technique and herein we describe an approach using preparative high performance liquid chromatography (HPLC) combined with cryoprobe-NMR to gain a reliable insight into the levels of metabolites, relative to parent drug, in the circulation of humans from early Phase I studies. Given that the parent drug concentrations in human plasma are almost always explicitly known, because of the analysis of parent drug for pharmacokinetic evaluation, an estimate can then be made of the concentrations of the major metabolites. With these data, therefore, it is not only possible to identify any metabolite, including those which may be unique to human, but also to compare the circulating concentrations of each metabolite in human with those found during metabolism studies in animals. As an early prelude to an HRS, this approach can be easily applied to both single and repeat dose regimens using relatively little resource and requires only the plasma that remains once the parent drug has been quantitatively analysed. Such an approach provides an early opportunity to assess the contribution that metabolites may make to the safety and tolerability of a drug-candidate, in advance of larger clinical trials, and in accordance with the FDA guidance [15]. This approach has been applied to a number of compounds and the determination of metabolite levels in human plasma are described here, for two experimental drugs.

2. Experimental

2.1. Chemicals and materials

HPLC grade acetonitrile, methanol and dimethyl formamide (DMF) were obtained from Fisher Scientific (Loughborough, UK). Analytical grade ammonium acetate was purchased from BDH (Poole, UK). 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). GSK-1 and five metabolites (M1, M2, M3, M4 and M5), GSK-2, [$^{2}H_{9}$]-GSK-2, and a metabolite (M6), and GSK-3, [$^{15}N^{13}C_{7}$]-GSK-3 and metabolites M7 and M8 were synthesised by GlaxoSmithKline Research and Development, Stevenage, UK. Blank human plasma was provided by GlaxoSmithKline Research and Development, Ware, UK.

2.2. Evaluation of methodology

2.2.1. Preparation of spiked human plasma

Blank human plasma was spiked with known concentrations of GSK-1 and its metabolite standards (M1–M5) at appropriate concentrations to mimic a sample typically generated from a clinical study. Stock solutions of GSK-1 (1 mg/ml) and M2 (1 mg/ml) were prepared in acetonitrile and the remainder, M1 (0.5 mg/ml), M3 (1 mg/ml), M4 (0.5 mg/ml) and M5 (1 mg/ml) were prepared in acetonitrile:de-ionised water (1:1, v/v). These were spiked into blank human plasma to provide a 100 ml plasma sample containing a range of concentrations: GSK-1 (2.5 μg/ml), M1 (0.5 μg/ml), M2 (5.0 μg/ml), M3 (2.5 μg/ml), M4 (15 μg/ml) and M5 (1.0 μg/ml), corresponding to approximately 5.5, 1.7, 15.7, 13.1, 52 and 4.0 μM respectively.

2.2.2. Preparative HPLC (GSK-1)

An aliquot (10 ml) of spiked human plasma was treated with 4 volumes of acetonitrile and vortex mixed (3 min). The resulting precipitate was centrifuged (1200 × *g* × 10 min) and the supernatant was aspirated and dried down using heated nitrogen at 37 °C. The residue was dissolved in methanol (1 ml) and reconstituted further by the addition of de-ionised water (9 ml). The entire sample (10 ml injection) was separated by preparative HPLC with fraction collection using a Waters 2767 inject/collect autosampler coupled to a 2525 binary pump and a 2996 photodiode array (Waters, Milford, USA). Separations were carried out using a Clipeus C18, 5 μm preparative HPLC cartridge column (100 mm × 10 mm i.d., Higgins Analytical, Presearch Ltd, Hitchin, Hertfordshire, UK) at ambient temperature with a mobile phase composition of 50 mM ammonium acetate and an acetonitrile gradient at a flow rate of 4 ml/min. The proportion of acetonitrile was programmed at 5% for 0–5 min, with an increase to 10% at 10 min, and further increases to 25% at 20 min and 45% at 30 min, with a final ramp to 100% at 35 min. HPLC eluent was collected into fractions, in a time-slice mode, into two 96 deep well plates using a frequency of 15 s per fraction. This resulted in 140 fractions, each containing 1 ml of column eluent. System control was mediated through MassLynx™ and FractionLynx™ (Waters, Milford, USA). The fractions were taken to dryness under nitrogen at 37 °C within the 96 deep well plates 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 5 mm NMR tubes.

2.2.3. NMR quantification (GSK-1)

NMR experiments were performed using a Bruker DRX-600 spectrometer equipped with an inverse 5 mm TXI CryoProbe™ ($^{1}H/^{13}C/^{15}N$) operating at 600.13 MHz under the control of XWIN-NMR (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 256 transients were acquired into 64 K 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. Routinely, 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. Prior to Fourier transformation, an exponential line broadening function of 1 Hz was applied to each spectrum to improve the signal-to-noise ratio. Appropriate peaks were quantified using the proton integration feature of XWIN-NMR software.

2.3. Analysis of samples from clinical studies (GSK-2 and GSK-3)

2.3.1. Clinical studies

Individual studies were conducted to assess the safety and tolerability of GSK-2 (20 mg) and GSK-3 (300 mg) in human volunteers following single and repeated daily administration respectively. Following administration of GSK-2, serial blood samples were taken from 12 volunteers at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 18 and 24 h post-dose. On the last day of GSK-3 administration, serial blood samples were taken from nine volunteers at 0, 0.5, 1, 2, 4, 8, 12 and 24 h post-dose. The derived plasma from each study were separately pooled across time points and subjects using the time-adjusted method described by Hop et al. [20] to give a large plasma pool. The two plasma pools were treated with 4 volumes of acetonitrile and then vortex mixed for approximately 3 min. The resulting precipitates were centrifuged (1200 × *g* × 10 min), and the supernatants aspi-

rated and then dried down under heated nitrogen at 37 °C. The dried residues were dissolved in *ca.* 50 ml aqueous methanol (v/v, 10%).

2.3.2. Preparative HPLC and NMR

The reconstituted extracts (*ca.* 50 ml) were separated by preparative HPLC using either a Waters 2767 or an Agilent series 1100 Preparative-LC system (Waldbronn, Germany). The Agilent system used an additional 1100 pump as a loading pump to apply the large volume plasma extract (50 ml) to the column [21]. Separations were carried out on either a Zorbax SB-C18 (GSK-2) 5 μm preparative HPLC cartridge column (50 mm × 21.2 mm i.d., Crawford Scientific, Lanarkshire, UK) or a Clipeus C18 (GSK-3), 5 μm preparative HPLC cartridge column (100 mm × 10 mm i.d., Higgins Analytical, Presearch Ltd, Hitchin, Hertfordshire, UK) at ambient temperature with a mobile phase composition of 50 mM ammonium acetate and an acetonitrile gradient, at a flow rate of 20 ml/min or 4 ml/min, respectively. For GSK-2 the proportion of acetonitrile was initially programmed at 20%, with an increase to 35% at 25 min, then a further increase to 70% at 30 min and finally maintained at 100% for 31–35 min. For GSK-3 the proportion of acetonitrile was programmed at 0% for 0–3 min, with an increase to 50% at 33 min, and finally maintained at 100% for 33–35 min. HPLC fractions were collected in the time-slice mode, over 35 min, into 12 mm test tubes using a frequency of 15 s per fraction to generate 140 fractions. System control was mediated through either MassLynx™ and FractionLynx™ (Waters, Milford, USA) or Chemstation™ (Agilent, Waldbronn, Germany). Each fraction was dried down under nitrogen at 37 °C and reconstituted in approximately 0.6 ml of deuterium oxide:acetonitrile (1:1) before being transferred to 5 mm NMR tubes. NMR analysis was carried out as described above. Results based on NMR were compared with those obtained using validated LC/MS/MS assays, for both GSK-2, GSK-3 and metabolites as described below.

2.3.3. Preparation of standards for LC/MS/MS quantification

Stock solutions of GSK-2 (1 mg/ml), metabolite M6 (1 mg/ml) and internal standard [$^{2}H_{9}$]-GSK-2 (0.1 mg/ml) were prepared in DMF. These were further diluted in acetonitrile:de-ionised water (1:1) to provide standard working solutions. Duplicate calibration standards containing GSK-2 [range 1–1000 ng/ml] and M6 [range 10–10,000 ng/ml] were prepared by spiking blank human plasma. Quality control (QC) samples were prepared independently, from a separate weighing of compounds, by spiking batches of blank plasma at 4, 50 and 800 ng/ml and 40, 500 and 8000 ng/ml for GSK-2 and M6, respectively. The internal standard working solution was prepared in acetonitrile at 2 ng/ml.

Stock solutions of GSK-3, metabolite M7 and metabolite M8 (1 mg/ml) and internal standard [$^{15}N^{13}C_{7}$]-GSK-3 (1 mg/ml) were prepared in methanol. These were further diluted in methanol or acetonitrile to provide standard working solutions. Duplicate calibration standards containing GSK-3 [range 10–15,000 ng/ml] and M7 and M8 [range 10–15,000 ng/ml] were prepared by spiking blank human plasma. Quality control (QC) samples were prepared independently, from a separate weighing of compounds, by spiking batches of blank plasma at 40, 200 and 12,000 ng/ml. The internal standard working solution was prepared in acetonitrile at 150 ng/ml.

2.3.4. Sample preparation

For both GSK-2 and GSK-3 aliquots (50 μl) of calibration standards, blank plasma, QC samples and plasma samples were individually dispensed into Eppendorf tubes and mixed thoroughly with the relevant internal standard working solution (150 μl) or equivalent amounts of acetonitrile for the blank plasma samples. Tubes were vortex mixed (10 min) and centrifuged (3000 × *g* × 20 min).

2.3.5. LC/MS/MS

All 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) or a Symbiosis (Spark Holland, Emmen, NL) coupled to an API-3000 or 4000 triple quadrupole mass spectrometer controlled through Analyst™ and equipped with a TurboIonspray® (TISP) source (Applied Biosystems, Toronto, Canada). Aliquots of plasma extract were separated using either a Hypersil GOLD, 5 μm column (50 mm × 4.6 mm i.d., ThermoHypersil, Cheshire, UK) or an Aquasil C18, 5 μm column (20 mm × 2 mm i.d., ThermoHypersil, Cheshire, UK) for GSK-2 and GSK-3 samples respectively, using a mobile phase composition of 10 mM ammonium acetate (A) and acetonitrile (B) at 1 ml/min. The Hypersil GOLD column was operated under isocratic conditions (A:B, 35:65, v/v), while separations on the Aquasil C18 column employed a step gradient. The proportion of acetonitrile was programmed at 5% for 0–0.3 min, and then increased to 95% at 0.4–1.0 min. The flow from both columns was directed to a TISP interface operating in the positive-ion mode (500–600 °C, argon collision gas setting 3). Analytes and internal standard 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). Calibration curves were constructed by plotting peak area ratios of analytes to internal standard against concentration and then calibration lines fitted using a weighted ($1/x^2$) linear regression model for all analytes. Concentrations of the analytes in plasma and the QC samples were interpolated from these plots. The concentrations were plotted against time and the area under the plasma concentration-time curve (AUC), for GSK-2, GSK-3 and metabolites, from just prior to dosing to 24 h post-dose (AUC_{0-24h}) were determined by non-compartmental analysis using WinNonlin™ (Pharsight, CA, USA).

3. Results and discussion

3.1. Initial evaluation

Herein we describe the application of preparative HPLC combined with cryoprobe-NMR for the quantification of drug metabolites in human plasma using methodology that is now routinely employed in the authors' laboratory. An overall schematic of the process is depicted in Fig. 1. The approach described takes advantage of the universal detection afforded by ^{1}H NMR; all components containing protons will be detected, with the proviso that there is sufficient sensitivity. The NMR analysis of drug metabolites in human plasma has become possible through the advent of cryoprobe-NMR, a technology which provides up to 4-fold increase in sensitivity over conventional NMR [18,19]. Furthermore, a spectrum which needed 16 h of acquisition time on a conventional NMR instrument can now be acquired within 1 h. Unfortunately, direct analysis of plasma for drug metabolites is not routinely possible by NMR due to significant endogenous interference, and some degree of sample separation is required. Historically solid-phase extraction (SPE) and liquid–liquid extraction (LLE) have been used for preparing biological samples for NMR [22,23]. However, the resolution of these approaches can be limited and the techniques are rarely capable of providing isolated fractions adequate for metabolite identification (and quantification in this case), without the intervention of a secondary clean-up technique. In contrast, prepar-

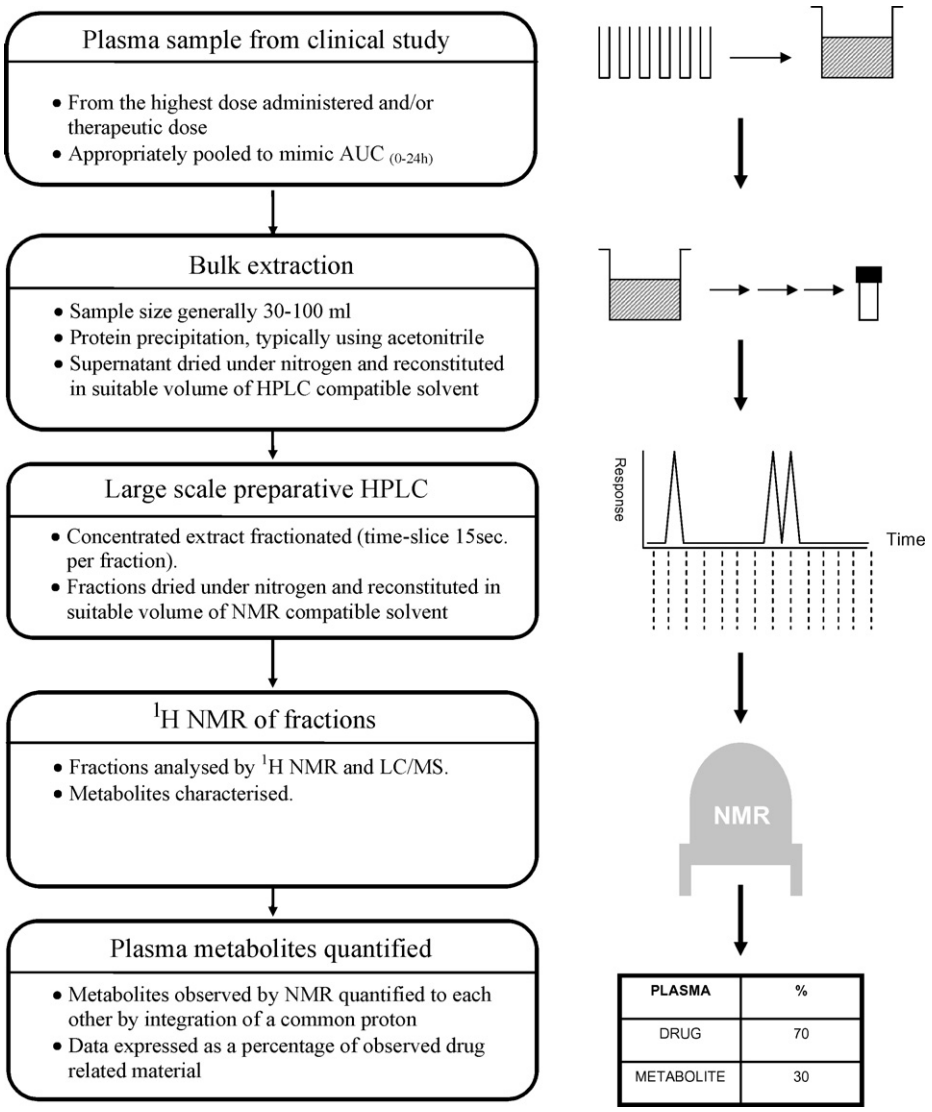


Fig. 1. Outline of process undertaken to identify and quantify drug metabolites in human plasma using preparative HPLC and cryoprobe-NMR.

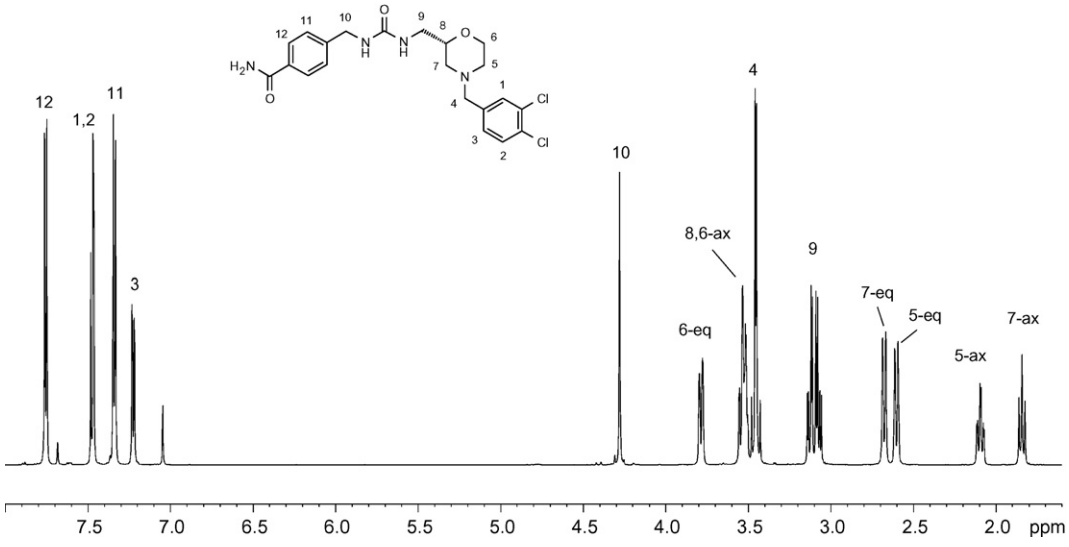


Fig. 2. ^{1}H NMR spectrum of authentic GSK-1 (in acetonitrile-d_3:deuterium oxide, 1:1).

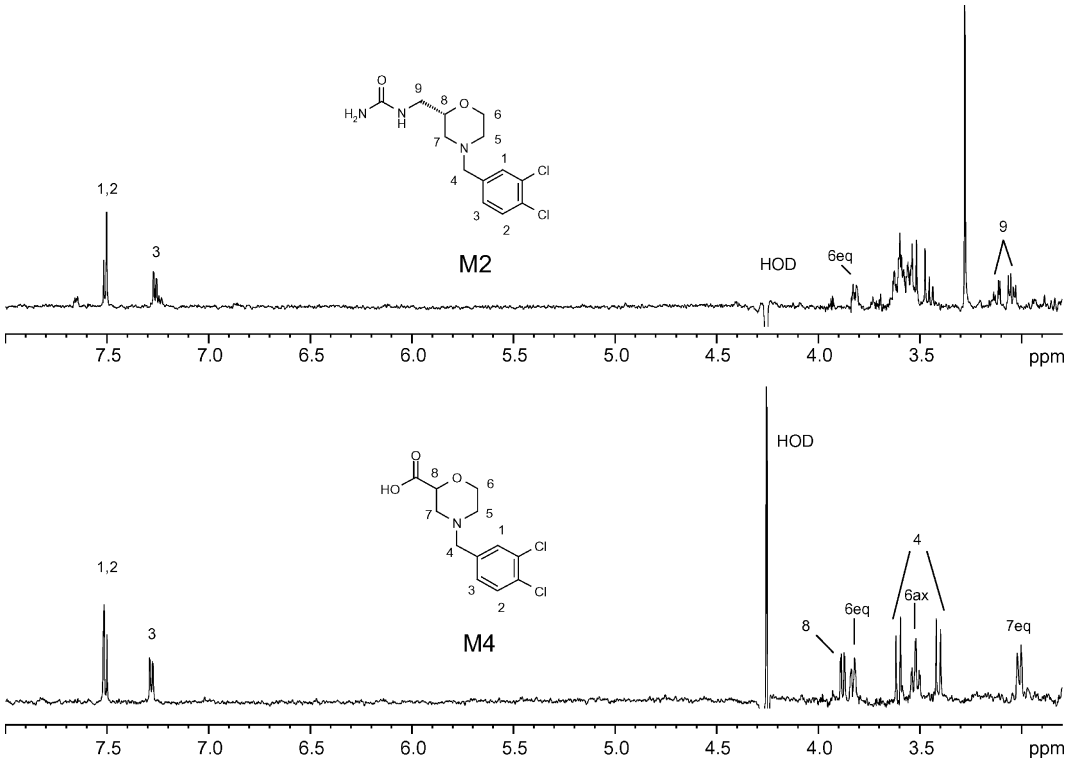


Fig. 3. Representative ^{1}H NMR spectra of GSK-1 metabolites, M2 and M4, isolated from human plasma.

ative HPLC has a large loading capacity and exhibits sufficient chromatographic resolution to isolate drug metabolites from complex biological samples. These separations are routinely performed in the gradient mode on relatively narrow diameter preparative columns (7.8–21 mm i.d.) with small particle sizes (5–7 μm) and are capable of isolating sufficient amounts (0.5–1000 μg) in a suitably clean state for this application. The chromatographic isolates are essentially free from salt, thereby affording the best sensitivity gain from the cryoprobe.

To evaluate the methodology, spiked control human plasma containing known levels of a drug candidate (GSK-1) and five metabolite standards was prepared. These were separated by preparative HPLC and all fractions were examined by ^{1}H NMR spectroscopy for drug-related material, as outlined in Fig. 1. The ^{1}H NMR spectrum of authentic GSK-1, 4-[({[({(2*S*)-4-[(3,4-dichlorophenyl)methyl]-2-morpholinyl}methyl)amino]carbonyl}amino)methyl]benzamide (Fig. 2) was used to assist the assignment of drug and metabolite proton resonances in the derived HPLC fractions. The ^{1}H NMR

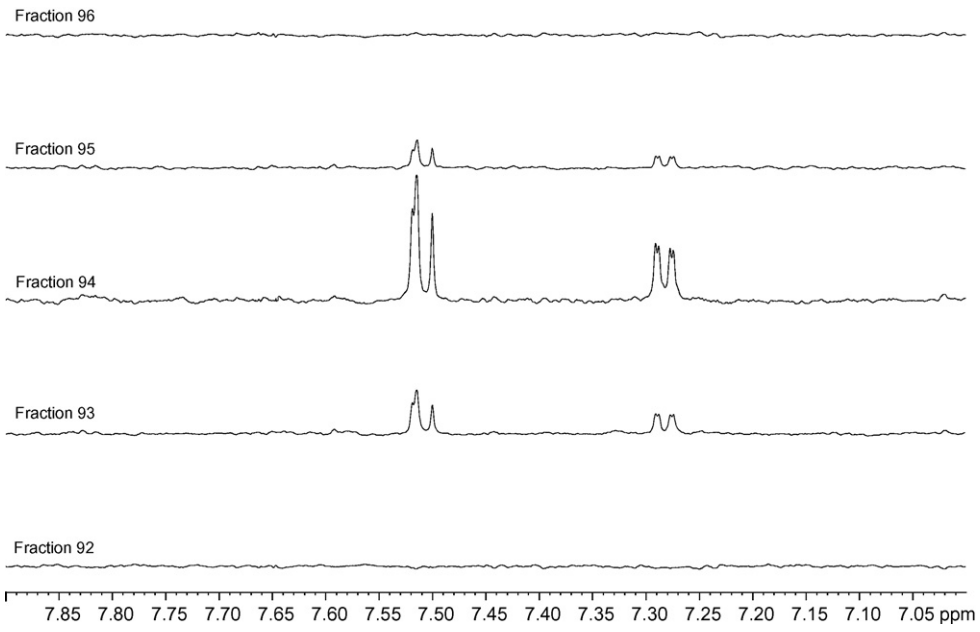


Fig. 4. Expanded ^{1}H NMR spectra of HPLC fractions 92–96, illustrating the elution of GSK-1 metabolite, M4.

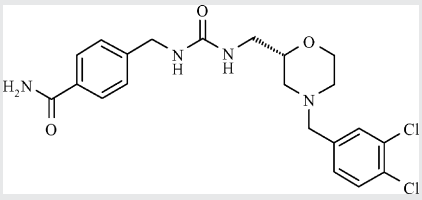
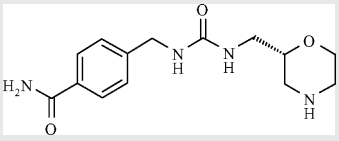
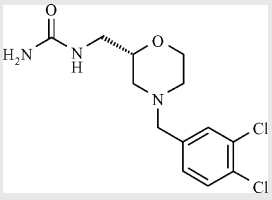
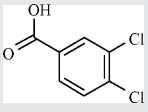
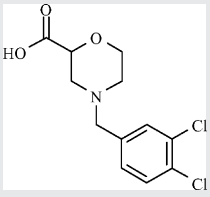
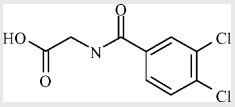
Table 1
^{1}H NMR integrals for a common proton from drug related material in fractions isolated by preparative HPLC from a spiked plasma sample.

Metabolite	Fraction no.	Integral
GSK-1	120	6
	121	9
M1	52	4
M2	115	7
	116	45
	117	3
M3	85	12
	86	15
	87	5
M4	93	35
	94	89
	95	19
M5	85	12

of GSK-1 was assigned as follows: δ: 7.75 (2H, d, J = 8 Hz, H-12), 7.48 (1H, d, J = 8.4 Hz, H-2), 7.46 (1H, d, J = 2 Hz, H-1), 7.34 (2H, d, J = 8 Hz, H-11), 7.23 (1H, dd, J = 8.4 Hz, 2 Hz, H-3), 4.28 (2H, s, H-10), 3.79 (1H, m, H-6eq), 3.55 (1H, m, H-6ax), 3.52 (1H, m, H-8), 3.48,3.44 (1H each, d, J = 13.4 Hz, H-4), 3.13 (1H, dd, J = 14 Hz, 5 Hz, H-9), 3.08 (1H, dd, J = 14 Hz, 6.6 Hz, H-9), 2.68 (1H, m, H-7eq), 2.60 (1H, m, H-5eq), 2.09 (1H, dt, J = 11.6 Hz, 3.8 Hz, H-5ax), 1.84 (1H, t, J = 10.9 Hz, H-7ax). Partial ^{1}H NMR spectra corresponding to the dealkylated metabolites M2 and M4, are shown in Fig. 3 and despite some endogenous material present in the samples, the drug-related resonances can be readily distinguished.

Relative amounts of each metabolite and parent drug can be estimated by comparison of the NMR integral of a common proton in the spectra and this is simply conducted by selecting a proton for quantification which is neither affected metabolically nor coinciding with endogenous signals. Preferably, an identical proton resonance should be selected for integration for each metabolite, to

Table 2
Comparison of the nominal and measured concentrations of GSK-1 and its metabolites in a spiked plasma sample.

Plasma component	% DRM (nominal)	% DRM (measured)
GSK-1 	6.0	5.8
M1 	1.9	1.6
M2 	17	21[a]
M3 	14	12
M4 	56	55
M5 	4.4	4.5

DRM–observed drug-related material.
[a] Minor unresolved endogenous peak affected integration.

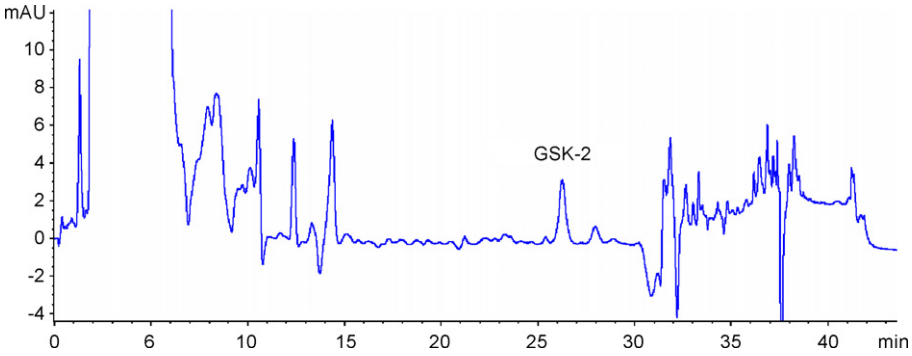


Fig. 5. Preparative HPLC-UV chromatogram (254 nm) of pooled plasma, following the administration of GSK-2.

minimise any effects due to insufficient T_1 relaxation. Alternatively, in cases where this is not possible a conservatively long relaxation delay can be applied. At all times sufficient acquisition time should be applied to obtain a signal:noise ratio of approximately 5:1 to ensure adequate integration. Although many NMR applications make use of flow-NMR [24] this approach is less suited to quantification of samples since some of the resultant drug-related fractions may require further data acquisition to increase the signal to noise. With flow-NMR there is invariably some degree of sample dilution with the transfer of a sample from plate to NMR probe and back. This dilution would have an unfavourable impact on the quantification step described in this method.

Once fractions containing GSK-1 and its metabolites were established, a more detailed assessment of drug and metabolite quantities was therefore obtained and the integrals for a common proton in each relevant fraction are shown in Table 1. Based on a comparison with parent drug, resonances at 7.75 or 7.48 ppm were integrated for each metabolite.

The process outlined in Fig. 1 produces discrete fractions which may contain drug and/or metabolites, together with co-eluting or partially co-eluting endogenous components. In the authors' experience a time-slice regime of 15 s per fraction (*ca.* half the typical peak width) represents a suitable compromise between maintaining some degree of chromatographic resolution while limiting the number of fractions generated. Drug related components typically elute in more than one sequential fraction and this must be accounted for when the spectral regions are integrated so that the integrals are an accurate measure of relative quantities. This is illustrated in Fig. 4 where M4, a metabolite of GSK-1, is visible in fractions 93–95, and can be seen to increase and then decrease, as it is eluted and collected across these fractions.

Using these values the total areas integrated for GSK-1 and each metabolite were expressed as a percentage of the total drug related material measured (%DRM, Table 2) and both the nominal and measured %DRM were shown to be in good agreement. However, a difference of 23% was observed for M2 due to the presence of an endogenous component with an interfering proton signal. This highlights the care needed in the selection of a common proton that is free from interfering resonances. This is easily assessed through resolution enhancement of the NMR resonance of relevant peaks using Gaussian multiplication.

This initial evaluation has demonstrated that preparative HPLC combined with cryoprobe-NMR is a viable approach for generating reliable information on the circulating levels of metabolites in human plasma.

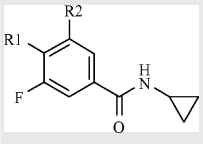
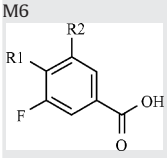
3.2. Analysis of clinical samples (GSK-2)

Following the initial evaluation described above (Fig. 1), the approach was applied to GSK-2, which was administered in a dose ascending clinical safety and tolerability study. In addition, the relative quantities of human circulating metabolites were corroborated by a subsequent validated LC/MS/MS assay, once suitable authentic metabolite standards became available. This served to further validate the approach described in this work.

To ensure sufficient material for NMR analysis, it proved necessary to pool samples to maximise the amount of drug-related material available. Sufficiently large volumes of plasma (30–100 ml) can readily be generated by using a time and volume adjusted pooling strategy across a number of subjects. The combined sample should reflect the relative proportions of the drug and its metabolites therefore providing a suitable understanding of the human exposure to these components. Hop et al. [20] found that the analysis of a single pooled sample of dog plasma (0–24 h) from a number of animals where the plasma volume pooled at each time point was dependent on the time difference between adjacent sampling times, gave similar apparent exposure, as measured by the area under the curve (AUC), to that obtained by LC/MS/MS analysis of individual samples.

For this study a large time-adjusted plasma pool was derived from 12 subjects following oral administration of GSK-2 (20 mg) and was extracted and fractionated in a similar manner to that described above, and depicted in Fig. 1. The resulting UV chromatogram following separation of pooled plasma is shown in Fig. 5 and illustrates the complexity of the sample matrix. Based on retention time comparison with authentic GSK-2, unchanged parent drug was assigned in this chromatogram. ^{1}H NMR of the resulting 140 HPLC fractions was firstly used to assist in the identification of

Table 3
Relative proportions of GSK-2 and its metabolites in human plasma taken from a single dose clinical study using NMR and subsequently confirmed using LC/MS/MS methodology.

Plasma component	% DRM (NMR)	% DRM (LC/MS/MS)
GSK-2 	26	31
M6 	74	69

DRM—observed drug-related material.

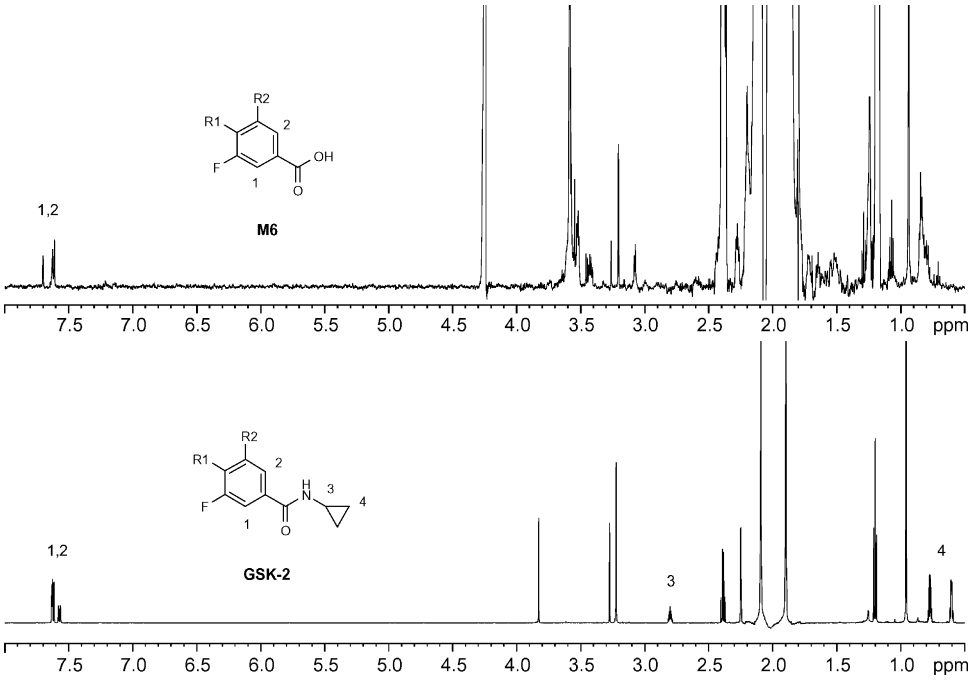


Fig. 6. Representative ^{1}H NMR spectra of GSK-2 and its metabolite, M6, isolated from human plasma.

drug-related material present in human plasma. Complementary LC/MS of selected HPLC fractions was also used to aid structural characterisation. Parallel to this activity, identification of GSK-2 metabolites in human plasma was also undertaken by conventional LC/MS [16], to supplement the NMR based approach. Based on an initial assessment of the NMR fractions, only two notable drug-related components were present. Unchanged parent drug was confirmed, through comparison with the NMR (Fig. 6) and mass spectra of authentic standard (*m/z* 384). The second component produced a protonated molecular ion at *m/z* 345, consistent with amide cleavage of the parent drug, to produce a carboxylic acid metabolite (M6). The associated NMR spectrum (Fig. 6) confirmed these findings. Following metabolite identification, the relative proportions of parent drug and the carboxylic acid metabolite (M6) were derived based on the integration of a relevant proton resonance in the corresponding HPLC fractions (Table 3). Subsequent synthesis of authentic M6 enabled the amount of GSK-2 and the metabolite, as determined by NMR to be compared with equivalent AUC values derived using a conventional LC/MS/MS assay (Table 3). The data showed that M6 was present at 2–3 times the level of GSK-2 and values obtained by both techniques compare favourably.

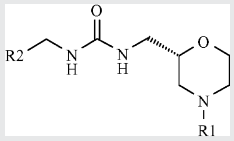
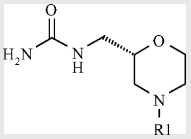
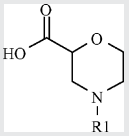
These results show that the accuracy of the preparative HPLC and cryoprobe-NMR approach is sufficient to guide the next step in development with respect to assessing a drug candidate's safety and efficacy. In this example, the formation of M6 was only a minor route of metabolism in the toxicology species and incubations of parent compound with human hepatocytes suggested that this metabolic route would likely be dominant in human (data not shown). By means of the NMR results presented here, M6 was confirmed as the predominant metabolite in human plasma and when compared with the radiometric estimates in rat and cynomolgus monkey plasma, it was considered likely that there had been inadequate exposure to M6 in the toxicology studies. By applying this approach, the information justified the need to quantify the metabolite in future clinical and preclinical studies to evaluate the requirement to dose the metabolite in subsequent toxicology studies.

3.3. Analysis of clinical samples (GSK-3)

In another example, plasma (day 14) taken following multiple oral administrations of drug-candidate GSK-3 (300 mg), were again pooled in a time and volume adjusted manner. Applying the approach outlined in Fig. 1, two metabolites were notable in human plasma, along with parent drug. Unchanged parent drug was confirmed, through comparison with the NMR and mass spectra of authentic standard (*m/z* 451). NMR and MS were then used to characterize the isolated drug metabolites. Both metabolites were identified as cleavage products, by interpretation of the ensuing spectra. The first metabolite, M7 was characterised as an N-dealkylation metabolite, giving rise to a protonated molecular ion at *m/z* 318. The second metabolite (M8), *m/z* 290, was rationalised through further N-dealkylation of the urea linkage with subsequent oxidation, to generate a carboxylic acid metabolite. Both structures were confirmed by interpretation of the associated ^{1}H NMR spectra. Again, identification of GSK-3 metabolites in human plasma was also undertaken by conventional LC/MS [16], to provide complementary data.

Following metabolite identification, the relative proportions of parent drug, M7 and M8 were derived based on the integration of a relevant proton resonance in the corresponding HPLC fractions (Table 4). Subsequent synthesis of authentic M7 and M8 again enabled the amount of GSK-3 and these metabolites, as determined by NMR, to be compared with equivalent AUC values derived from a validated LC/MS/MS assay (Table 4). Again results generated by NMR are consistent with values from the validated assay. An interesting feature of this example is that M8 could not be detected or quantified via radiometric analysis in pre-clinical studies using [^{14}C] GSK-3, prior to any human metabolism investigations, due to the position of the radiolabel. Based on the pattern of hydrolysis observed here, M8 loses the radionuclide, and hence was invisible in the radiometric analysis. M8 is also a poor ionizer in the mass spectrometer, so in the absence of the NMR approach described, M8 could have potentially remained undetected, even following a definitive HRS.

Table 4
Relative proportions of GSK-3 and its metabolites in human plasma taken from a repeat dose clinical study using NMR and subsequently confirmed using LC/MS/MS methodology.

Plasma component	% DRM (NMR)	% DRM (LC/MS/MS)
GSK-3 	10	9
M7 	16	12
M8 	74	79

DRM—observed drug-related material.

3.4. General remarks

Not only have these data provided early human metabolism information to help guide further safety assessment and future clinical studies they have also aided the design of the definitive HRS. Indeed the authors' have found that this approach can offer an advantage and supplement the data from an HRS especially if the compound is cleaved and loses radiolabel from a substantial proportion of the drug-related material. Furthermore, the HRS is generally designed as a single dose study but a high proportion of drugs are administered on a daily basis and as demonstrated here, the NMR procedure can be performed on plasma (or blood, data not shown) from any clinical study, including repeat dose, as long as a suitable dose level has been administered or where sufficient sample is available. Based on the authors' experience with this approach, if the sample contains *ca.* 5 μg of parent drug this typically allows for any metabolite representing approximately 10% of parent drug levels, to at least be quantified, if not characterised. For a large plasma volume (e.g. 50 ml) this may correspond to metabolite levels as low as 10 ng/ml. This is consistent with FDA guidelines [15] which recommend that metabolites identified in human plasma that account for greater than 10% of parent drug should be considered for their impact on patient safety. This generally precludes this application for compounds which are low dose or inhaled, or where sample volumes may be limited. In general compounds that have been dosed at ≥50 mg have yielded valuable data to guide the next step of the drug development process.

Perhaps the drawback of the cryoprobe-NMR based approach is still the relatively low sensitivity of NMR in comparison to other analytical techniques such as LC/MS. While the combination of cryoprobe technology and large-scale sample preparation has given us the ability to analyse human plasma samples, the need for pooling of plasma to get sufficient material does mean that any insight into inter-subject variability is lost. Further sensitivity gains in NMR or the use of smaller diameter cryoprobes would allow for work on smaller samples or lower metabolite concentrations. In addition, no routine assessment of drug or metabolite recovery from plasma is afforded by this technique, although the extraction methodology is generally guided by experience from preclinical radiolabel studies, to minimise any recovery losses or potential degradation.

4. Conclusions

Investigations into drug metabolism in humans is generally achieved through dosing a radiolabel drug as part of a clinical study and this is typically conducted post-proof of concept in the clinical development plan. Using preparative HPLC and cryoprobe-NMR, as described, to determine the systemic exposure of drug metabolites using samples derived from Phase I clinical studies, provides an early understanding of human metabolism. Of the 25 drug candidates examined by this approach in the authors' laboratory, to date, 17 had metabolites that were adequately represented in one or more of the toxicology species. For the other 8 compounds insufficient preclinical exposure was demonstrated, such that in 4 examples, one or more metabolites required synthesis and dosing to animals prior to further evaluation in humans. This approach therefore provides an early opportunity to assess the contribution metabolites may make to the safety and tolerability of a drug-candidate, in advance of larger clinical trials, and in accordance with the FDA guidance.

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