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Research article

Harmonised high throughput microsomal stability assay

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ABSTRACT

Introduction: Prediction of human pharmacokinetics from *in vitro* assays and pre-clinical data is an integral part of drug discovery. *In vitro* stability metabolic studies can provide an estimate of *in vivo* hepatic intrinsic clearance through inclusion of biological scaling factors. Many labs have personalised stability protocols including marker compounds and have adopted QC criteria and assay limits to ensure data integrity. Contract research organisations (CRO's) provide integrated drug discovery support to academic and pharmaceutical/biotechnology institutions to progress their in-house projects. The majority of these clients have established in-house protocols with associated criteria to ensure data consistency between in-house and external labs.
Methods: In this study, numerous assay variables were condensed into one harmonised assay format and a range of compounds with diverse physicochemical properties were evaluated. The protocols were diverse with respect to the following attributes: buffer, microsomal concentration and species strain.
Results: Comparison of human lots *in vitro* CL_{int} between the traditional and consolidated assay formats showed a good correlation with no significant difference. A clear relationship was demonstrated between strains. Interpretation of *in vitro* intrinsic clearance between the strains for each species was consistent. Using strict classes of *in vitro* hepatic intrinsic clearance values (<50, 50–100, >150 μL/min/mg protein) comparisons across different conditions such as, assay variables, human lots, mouse and rat strains showed >80% agreement.
Discussion: A high throughput assay was developed that enables the simultaneous measurement of CL_{int} using mouse, rat and human hepatic microsomes (consolidated assay). By condensing all possible variables into one assay format, consistent data were obtained during head to head tests.

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1. Introduction

Over the past decade, pharmaceutical companies have introduced a number of new approaches dedicated to address pharmacokinetic (PK) attrition rates and increase the success of drug development (Kola & Landis, 2004). Screening in drug discovery has dramatically increased over the past years, positively correlating to the development of New Molecular Entities (NMEs) (Baranczewski et al., 2006). Increased capacity of tier 1 screens has led to heightened demands for early information on absorption, distribution, metabolism, excretion (ADME) properties (van de Waterbeemd & Gifford, 2003). The aim of this approach is to identify potential development liabilities of NCEs as early as possible, thereby minimising the risk of late failure of new drug candidates (Kassel, 2004).

One key *in vitro* strategy employed in the last decade is the early determination and prediction of metabolic clearance (Plant, 2004). The utilisation of high throughput *in vitro* methods for drug metabolism studies has several advantages: 1. Early determination of the metabolic profile which provides ranking capabilities and crucial information for drug metabolism and pharmacokinetics (DMPK) teams and medicinal chemists to guide further modifications thus obtaining more favourable metabolic properties; 2. Human enzymes, cells and liver fractions can be used in addition to pre-clinical species to predict human *in vivo* clearance from the onset, 3. The *in vitro* approach is cost- and time-effective in terms of ease of preparation from many species, long term storage and flexible availability (Riley, Martin, & Cooper, 2002). The failure of NMEs related directly to inadequate metabolic and PK parameters was reported to have fallen to approximately 10% in 2000 (Kassel, 2004) as a result, at least in part, of strategic investment in such enhanced throughput *in vitro* assays.

Methods to determine *in vitro* clearance of NMEs are now even more accessible and efficient due to advances in automated technology (Riley

Abbreviations: ADME, absorption distribution metabolism excretion; AFE, average fold error; CLint, intrinsic metabolic clearance; CRO, contract research organisation; CYP, cytochrome P450; DMPK, drug metabolism and pharmacokinetics; G6P, glucose-6-phosphate; t1/2, half-life; HTS, high throughput screening; DMSO, dimethyl sulfoxide; LC-MS, liquid chromatography mass spectrometer; NME, new molecular entities; NADPH, nicotinic adenine dinucleotide phosphate; PD, pharmacodynamic; PK, pharmacokinetic; QC, quality control.

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& Grime, 2004). These high throughput screening (HTS) protocols use microtiter plates (96-, 384- and 1536-well plate format) (Yasgar et al., 2010) for incubations, robotic systems for liquid transfer (*e.g.* Packard MultiPROBE® II, SAGIAN™ robotic system supplied by Beckman Coulter) and high capacity liquid chromatography mass spectrometer (LC-MS) detection methods (Di, Kerns, Ma, Huang, & Carter, 2008; Fonsi, Orsale, & Monteagudo, 2008; Jenkins et al., 2004; Kariv, Fereshteh, & Oldenburg, 2001; Taylor, Janisewski, & Whalen, 2006). With the advent of enabling technologies, most mid to large pharmaceutical companies have designed specific metabolic stability protocols using specific lots of microsomes, hepatocytes, internal quality controls (QCs) and acceptance criteria.

During the last decade the pharmaceutical industry has seen an increase in outsourcing drug discovery work, such as, fee-for-service ADME screens to CROs and entire discovery programmes as part of an integrated project workflow (Cavalla, 2003; Clark, 2007; Clark, 2011; Clark & Newton, 2004). CROs work with numerous clients ranging from small academic groups to large pharmaceutical companies. Prior to screening, validation analysis is completed with compounds covering a range of chemical series/physicochemical properties and metabolic stability to ensure data correlate with existing in-house values. Protocols to test metabolic stability in microsomes can vary greatly across companies and even between different sites within the same company. Variations can include but are not limited to: compound concentration, microsomal protein concentration, incubation time, pre-clinical species and strain, specific microsomal lots, molarity of nicotinamide adenine dinucleotide phosphate (NADPH), use of an NADPH regenerating system, buffer composition, incubation time, time points, analytical conditions, quality controls (QCs) and cut off criteria (Andersson, Bredberg, Ericsson, & Sjoberg, 2004; Austin, Barton, Cockroft, Wenlock, & Riley, 2002; Carlile, Hakooz, Bayliss, & Houston, 1999; Naritomi et al., 2001; Obach, 1999; Riley, Mcginnity, & Austin, 2005). QC and interpretation of depletion profiles have been the subject of significant previous reports (Jia & Liu, 2007), hence are not addressed specifically here.

Establishing and running many client-specific methods and variants on a weekly basis can be an extremely time-consuming process from preparation of assay set-up, robot programmes and analytical time thus limiting assay throughput and requiring regular investment in new equipment. To address these issues, the authors have optimised one high throughput protocol for testing microsomal stability in a range of species, deemed the consolidated HTS assay (throughout this paper the term “traditional assay” is used to define the multiple protocols used previously). In combination with additional ADME and potency assays, compounds with high microsomal turnover may not be appropriate for further progression as potential once-daily oral drugs but those displaying promising stability may be advanced into further stability assays, including a relay assay or a plated low clearance assay (Bonn, Svanberg, Janefeldt, Hultman, & Grime, 2016; Di et al., 2012). In the current study, 108 literature and project compounds were evaluated exhibiting diverse chemical scaffolds and physiochemical properties to assess *in vitro* metabolic clearance. Understanding stability in numerous species is required for constructing *in vitro-in vivo* extrapolations which will ultimately be applied to estimate human PK and dose. The main aims of this study were two-fold: to determine if variables from diverse protocols could be condensed into one HTS assay and; to confirm if this assay was able to provide comparable, fit-for-purpose screening data across human microsomal lots and strain for pre-clinical species.

2. Materials and methods

2.1. Materials

Microsomes (Wistar rat) were purchased from Xenotech/Tebu-bio (Peterborough, UK). Microsomes (human 10 donor; Lots UIK, YBU) were purchased from Bioreclamation (West Sussex, UK). Microsomes (SD rat, CD-1 mouse and C57/BL6 mouse) were purchased from BD Biosciences (Oxford, UK). All other chemicals and materials were purchased from Sigma-Aldrich (Dorset, UK).

3. Methods

3.1. NCE preparation

Solubility of the NMEs in the consolidated assay was improved through inclusion of an additional dilution step. The traditional assay involved the dilution of 250 μM (50% DMSO: 50% H_2O) directly into medium to achieve a final concentration of 1 μM. The consolidated assay involved an additional dilution to 100 μM (91.5% acetonitrile: 8.5% DMSO) before dilution to 1 μM in the assay medium. The use of a vehicle mostly composed of acetonitrile also limits the impact on some cytochrome P450 (CYP) substrates in some species (Riley & Grime, 2004). Further, 1 μM was selected as an appropriate concentration to achieve first-order depletion. Whilst not all compounds display first-order kinetics determination was beyond the scope of this work and beyond the capacity of HTS tier 1 screening.

Multiple projects were evaluated to ensure inclusion of compounds with a range of physico-chemical properties ($LogD_{7.4}$ 0.5–>4.5), chemotypes (acid, base, neutral) and oxidative metabolic pathways.

3.2. Microsomal incubations

A pragmatic approach based on collation of most common client practises/needs and literature data was applied rather than a systematic head-to-head analysis of all variables described in Fig. 1. For examples, previous studies have demonstrated that selection of microsomal protein concentration will be a balance of turnover, non-specific microsomal binding and cost (Austin et al., 2002). Tier 1 microsomal screening requires coverage of a reasonable dynamic range of stability values to discriminate between compounds. The consolidated assay included known literature controls to cover low, moderate and high clearance values (Table 1).

The microsomal incubations were prepared in 96-well plates by a Perkin Elmer Janus Robot to a final volume of 500 μL (incubation mixtures were as described in Fig. 1). Following a pragmatic approach based on most common client practises and literature data, concluding incubation mixtures consisted of: 1 μM test compound, liver microsomes (0.5 mg microsomal protein/mL), 2 mM $MgCl_2$, and 25 mM potassium phosphate buffer, pH 7.4, in a final volume of 500 μL. All incubations were conducted in duplicate. Reactions were initiated through the addition of NADPH by the Janus Robot (final concentration

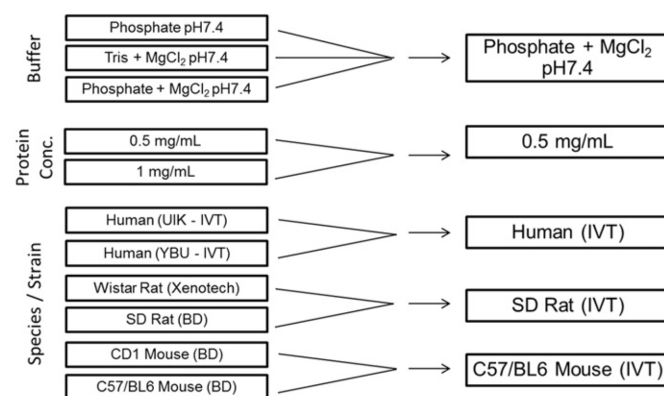

Fig. 1. Variations of the traditional assay converged into one consolidated HTS assay. The traditional assay involved multiple combinations of the first column which were subsequently condensed based on a pragmatic approach of the most common client practises/needs into the consolidated HTS assay.

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