CHALMERS
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Miniaturization of two high throughput metabolic stability assays in early drug discovery

Master's thesis in Biotechnology

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Department of Biology and Biological Engineering
CHALMERS UNIVERSITY OF TECHNOLOGY
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Abstract

When it was recognized that evaluation of pharmacokinetic (PK) properties during drug discovery strongly influenced the success rate during clinical development, ADME (Absorption, Distribution, Metabolism, Excretion) properties gained more and more attention in the pharmaceutical industry. One of the important PK properties to investigate is metabolic clearance since it directly relates to drug elimination and bioavailability. This master's thesis project was performed at AstraZeneca where *in vitro* metabolic stability is screened in early drug development using human liver microsomes and cryopreserved rat hepatocytes. The assays are run in 96-well format and the aim of this project was to miniaturize the two assays to 384-well format. Different equipment was tested when performing the experiments and the incubation volumes were optimized. The developed assay using human liver microsomes was validated in two steps. First, intra-assay and inter-assay variability was determined and the assay was compared with the existing 96-well format assay. The resulting data showed that AFE was 1.30 and AAFE 1.33. Then, the data was used to investigate the *in vivo* prediction and the results showed that the data align well with *in vivo* clearance data. The CL_{int} values obtained from the developed assay using hepatocytes were on average lower than the CL_{int} values obtained from the existing assay. Adding shaking decreased AFE from 2.19 to 1.29 and AAFE from 2.52 to 1.62 but seven of the 19 tested compounds did still have an average fold change ≥ 2. Lack of sufficient mixing during incubation was probably the main reason.

Keywords: Metabolic stability, CL_{int}, human liver microsomes, rat hepatocytes, IVIVE

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Beatrice d'Aubigné, Gothenburg, June 2018

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Introduction

ADME (Absorption, Distribution, Metabolism, Excretion) properties of drugs have gained more and more attention in the pharmaceutical industry and early screening of new chemical entities now generally includes high-throughput ADME measurements [1]. The movement towards early ADME screening began when it was recognized that evaluation and optimization of pharmacokinetic (PK) properties during drug discovery strongly influenced the success rate during clinical development. Compound failures due to poor PK properties in the clinical phase decreased from 40% in 1990 to 10% in 2000, which was directly connected to the increased attention to PK evaluation during drug discovery. The early screening entails that a large number of compounds need to be tested and this has been enabled by advances in automated compound synthesis, high-throughput analytical technologies and automated assays. Since it is not possible to perform *in vivo* experiments at this early stage for this high amount of compounds the use of *in vitro* ADME assays plays an important role [2].

Metabolic clearance (CL) is one of the more important PK parameters to study in drug discovery since it directly relates to drug elimination and bioavailability [3, 4]. Knowledge of clearance in humans makes it possible reject compounds that are likely to be eliminated too fast or too slowly from the body and instead focus on compounds with desired metabolic stability. Metabolic clearance is commonly studied *in vitro* by determining intrinsic clearance (CL_{int}) which is scaled up to predict *in vivo* CL and if the prediction is successful it could lead to significant savings in time, cost and animals [4].

1.1 Background

This master's thesis project was performed in collaboration with AstraZeneca, where *in vitro* metabolic stability of new chemical entities is evaluated for the first time in two assays that are part of the Wave 1 DMPK screening. Apart from metabolic stability the Wave 1 DMPK screening also support drug developent projects with data regarding compounds' solubility, logD and plasma protein binding. The data is used by the projects to do a first ranking of the compounds and to optimize the chemical structures to get favorable PK parameters.

All five Wave 1 assays are run weekly in 96-well format on a Hamiltion Microlab Star robot. The compounds that have been requested for testing are first ordered from compound management (CM) by the Wave 1 team. The goal is then to perform the assays and report the data within five days. Figure 1.1 shows an overview of the Wave 1 test process from when the test is requested to when the data is reported.

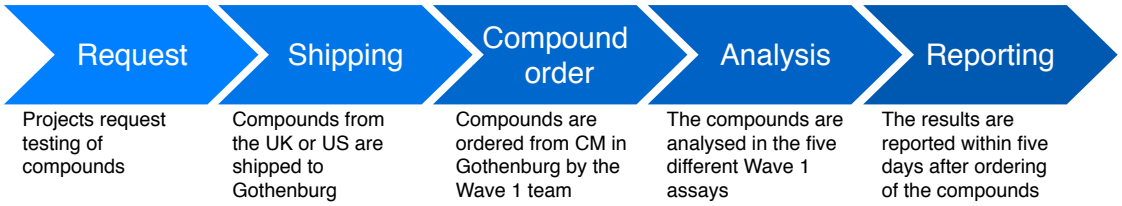


Figure 1.1: Overview of the Wave 1 test process.

Human liver microsomes are used in one of the Wave 1 metabolic stability assays and cryopreserved rat hepatocytes are used in the other. Both assays use serial sampling from the incubation plate at different timepoints and UPLC-MS/MS for analysis to determine the halflife ($t_{1/2}$) from which intrinsic clearance (CL_{int}) of the compounds is calculated. Sampling occurs at nine timepoints in the assay using hepatocytes and at six timepoints in the assay using microsomes. A maximum of three incubation plates can be used per run, resulting in that 288 compounds (282 test compounds and six control compounds) can be screened per week. The compounds are pooled together according to Figure 1.2 before analysis to shorten the analysis time on the UPLC-MS/MS. The experimental protocols of the two metabolic stability assays are presented in Appendix A.1.

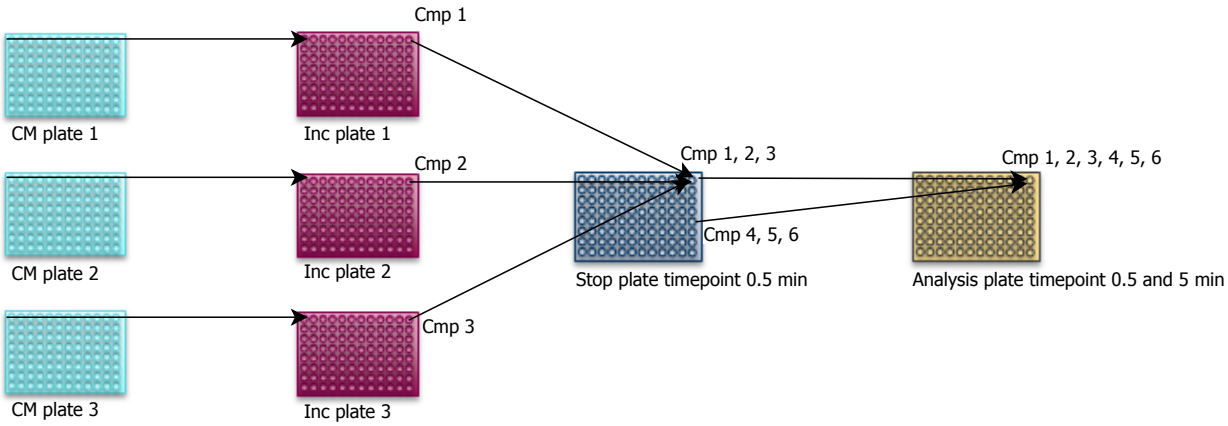


Figure 1.2: Summary of the incubation and pooling script on the Hamilton Microlab Star robot when three incubation plates are used. The figure shows the concept for only one timepoint. The assay using microsomes has seven stop plates (one for each timepoint and one for the blank plate) and four analysis plates. The assay using hepatocytes has ten stop plates (one for each timepoint and one for the blank plate) and five analysis plates. Six compounds are pooled together before the analysis on the UPLC-MS/MS when three incubation plates are used.

In the third quarter of 2018, CM will start using acoustic tubes and acoustic dispensing when distributing the compounds to the microplates. This is in line with

a more general goal to reduce the amounts of compound, cells and plastic that are used. The acoustic tubes will have a working volume of 70 µL and since that is less than today's vials, the transition from pipetting to acoustic dispensing has effects on the ordering volumes. To align the Wave 1 assays to acoustic dispensing, a goal to scale-down the Wave 1 assays to 384-format has been established. Regarding the metabolic stability assays, that already use rather low amounts of compound, the greatest benefit from the miniaturization would be the reduction of microsomes and hepatocytes used. In addition, miniaturized metabolic stability assays are believed to be more efficient as well as they could be used for screening more compounds per week.

1.2 Aim of project

The aim of this master's thesis project was to transfer two metabolic stability assays, one using human liver microsomes and the other rat hepatocytes, to 384-well format. To do this different equipment for performing the assay should be evaluated and the experimental protocols should be optimized. The aim was also to validate the developed protocols to determine if the new assays could deliver data of the same quality as the existing assays, which is necessary if the new assay format should replace the existing one.

2

Theory

2.1 Biotransformation of Xenobiotics

Xenobiotics are chemicals that an organism is exposed to that are extrinsic to the normal metabolism of that organism [5]. Examples of xenobiotics are pollutants, food additives and drugs. The lipophilicity of the xenobiotic is often what determines how easily it can be absorbed through the skin, the lungs or the gastrointestinal tract. When inside the body, lipophilic compounds are obstruct from being excreted because they are readily reabsorbed. Therefore, biotransformation of the xenobiotic occurs to increase the excretion rate by converting the chemical into a more water-soluble metabolite. Without metabolism, many xenobiotics would not be eliminated fast enough from the body to avoid toxic concentrations [6, 7].

Biotransformation reactions are divided into two groups, phase I and phase II reactions. Phase I reactions involves hydrolysis, reduction and oxidation of the compound and either introduce or unmask functional groups like -OH, $–NH_2$, -SH or -COOH. This transformation increases the hydrophilicity slightly which in some cases is enough for excretion. In other cases, the product that is formed also undergoes a phase II reaction to further increases the hydrophilicity [6, 7]. Phase II reactions are reactions of conjugation that require a suitable functional group on the substrate where the endogenous molecule or moiety such as CH_3, sulfate or glucuronic acid can be attached. The functional group is either present on the xenobiotic or it is created by the phase I reaction [7, 8].

Biotransformation of drugs and other xenobiotics most often starts with the phase I redox reactions and a majority of these reactions are catalyzed by cytochromes P450 (CYPs), even though other oxidoreductases like flavin-containing monooxygenases (FMOs) are important as well. CYPs are present in essentially all tissues in the human body but the expression levels differ much between different tissues and cells. In humans, the liver expresses the highest amount of these enzymes [5]. The CYP superfamily consists of over 11294 genes grouped into 977 gene families. 18 of these gene families are found in humans, with a total of 107 genes [5, 9]. CYPs have a broad and overlapping substrate specificity, however most of the human CYP genes have specific endogenous functions e.g. synthesis of steroid hormones and bile acids. Only about a dozen of the different CYPs are involved in drug and other xenobiotic

biotransformation. CYP3A enzymes are often considered the most important CYP enzymes in drug metabolism, since they play a major role in the biotransformation of ca. 30% of all clinically used drugs [10].

The most representative type of CYP-mediated phase I redox reaction that drugs undergo are the monooxygenation reactions. The monooxygenation reactions involve reduction of O_2 by two electrons, where one of the oxygen atoms are incorporated in the substrate while the other oxygen atom forms water. The two electrons involved are carried by two electron transport chains. The first electron is transferred to the CYP enzyme from NADPH by NADPH-cytochrome P450 oxidoreductase. The second electron can be transferred in the same way but it can also come from NADH via cytochrome b_5 [5, 6].

2.2 Metabolic stability studies

Biotransformation of drugs affects both their bioavailability and clearance, which are two major pharmacokinetic properties. The bioavailability of a drug is defined as the fraction of the administrated drug that reaches the systemic circulation unchanged. Factors that affect the bioavailability of orally administrated drugs are for example the absorption through the intestinal wall and first-pass elimination, i.e. the biotransformation that the drug undergoes before it reaches the systemic circulation. Clearance describes the volume of plasma that is cleared from the compound per unit of time, often measured in ml/min. The overall clearance of a drug is the sum of the renal, biliary, extrahepatic and hepatic clearance [11]. Since the liver is the most important organ for drug metabolism, hepatic metabolic clearance (CL_H) is often of primary importance during the discovery phase [4]. There exists several models that describe hepatic clearance and the three most applied once are the "well stirred", "parallel tube" and "dispersion" models. Common for all three models is that they are based on three basic parameters: intrinsic clearance (CL_{int}), free fraction of drug in blood and liver blood flow [12].

In vitro metabolic stability studies investigate the susceptibility of compounds to biotransformation and the results are generally expressed as *in vitro* half-life ($t_{1/2}$) and CL_{int}. As with many biological enzyme-based reactions, the Michaelis-Menten model is used for basic description of the kinetics. To calculate CL_{int} it is necessary that the ratio of metabolism is linear with incubation time, which can be assumed when the substrate concentration is lower than the Michaelis-Menten constant (K_m), see Figure 2.1. Since K_m values usually aren't determined at the time for the metabolic stability studies it is desirable to keep the substrate concentrations in the assay low but still at levels where the analytical method is sensitive [13].

The ratio of metabolism in metabolic stability studies is either determined by measuring metabolite formation or substrate depletion over time. The substrate depletion method is more widely used since it does not require knowledge about the metabolites that are formed. In this method, the natural logarithm of percent remaining of the parent compound is plotted versus time. The slope of the linear

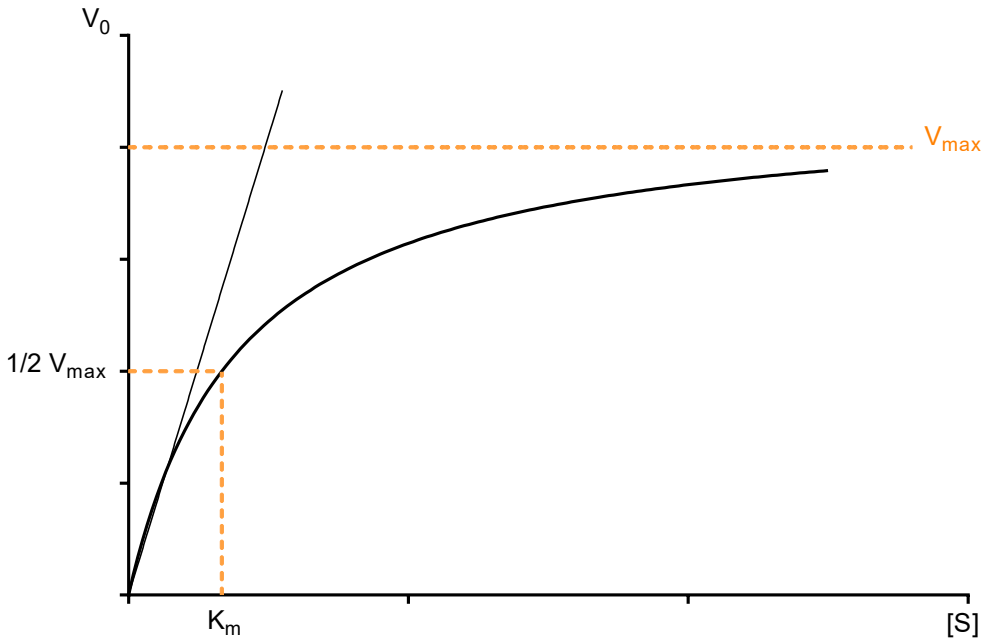


Figure 2.1: Plot of Michaelis-Menten enzyme kinetics where it is illustrated that if the substrate concentration [S] is lower than K_m the rate of metabolism is approximately linear.

regression fit gives the rate constant for the disappearance of parent compound (k) which is used to calculate $t_{1/2}$ and CL_{int} according to equation 2.1 and 2.2:

$$t_{1/2} = \frac{\ln 2}{k}(\text{min}^{-1}) \tag{2.1}$$

$$CL_{int} = \frac{k}{C_{protein}} = \frac{\ln 2}{t_{1/2} * C_{protein}}(\mu L/(mg * min) \text{ or } \mu L/(10^6 cells * min)) \tag{2.2}$$

Where $C_{protein}$ is the protein concentration in the incubation, measured in mg/µL if microsomes are used and in million cells/µL if hepatocytes are used [13].

2.2.1 Microsomes in metabolic stability studies

Microsomes are small spheres formed from the endoplasmic reticulum (ER) after homogenization of a tissue. They can be isolated from other compartments in the homogenized mixture using differential centrifugation. Microsomes are suitable to use in metabolic stability studies since the drug-metabolizing CYPs are found in highest concentration in the ER membrane and approximately 90% of all marked drugs and drug candidates are substrates of human CYPs [6]. This has, together with the fact that microsomes are cheap and easy to use and store, lead to that they have been the most commonly used *in vitro* system for metabolic stability

assays [6, 14]. However, microsomes lack phase II enzymes which affects the *in vitro* CL_{int} value of drugs to different extent, depending on how important secondary metabolism they exhibit. This has to be taken into account when predicting *in vivo* CL_{int} from the *in vitro* data [15]. The lack of phase II metabolizing enzymes can also lead to product inhibition because the metabolites formed from the phase I reactions are accumulated [16]. In addition, microsomes can only be incubated for a short time (1-2 hours) before they start loosing their activity which makes them unsuitable for monitoring CL_{int} of poorly metabolized drugs that would need longer incubation time [15].

2.2.2 Hepatocytes in metabolic stability studies

Hepatocytes are the predominant cell type in the liver and they are responsible for its metabolic function [9]. Since they are intact cells containing both phase I and phase II enzymes as well as transporter proteins they are considered a more accurate *in vitro* system than microsomes for metabolic studies. The limited availability and high cost of fresh hepatocytes, especially human, has however resulted in that cryopreserved hepatocytes are the favored alternative [14]. If thawed correctly, crypreserved hepatocytes show comparable metabolic enzyme activities as the fresh isolated hepatocytes [13].

2.3 *In vivo* predictions of *in vitro* CL_{int} data

As mentioned above, there exist several liver models for relating CL_{int} to CL_H [12]. The well stirred model is the most used model and the conventional version of the model can be seen in equation 2.3.

$$\text{Predicted } CL_H = \frac{Q_h * fu_{bl} * CL_{int\ \textit{in vivo}}}{Q_h + (fu_{bl} * CL_{int\ \textit{in vivo}})} \tag{2.3}$$

Where Q_h is the total liver blood flow, fu_{bl} is the fraction unbound in whole blood and $CL_{int\ \textit{in vivo}}$ is the *in vivo* intrinsic clearance. fu_{bl} is seldomly measured but can be calculated using equation 2.4.

$$fu_{bl} = fu_{pl} * \frac{C_{pl}}{C_{bl}} \tag{2.4}$$

Where fu_{pl} is the fraction unbound in plasma, C_{pl} is the plasma concentration and C_{bl} is the concentration in whole blood. Apart from the conventional form of the well stirred model, other *in vitro-in vivo* extrapolation (IVIVE) methods where the binding corrections are altered have been developed to improve the prediction of hepatic metabolic clearance. For example, one of the methods excludes the fu_{bl}

correction factor and another method adds the correction for unbound fraction in the incubation (fu_{inc}). The prediction performance of the different methods is connected to the drug properties, such as the extent of drug binding to albumin and blood and if the drugs show high or low clearance [3].

Apart from predicting CL_H from CL_{int} *in vivo*, CL_{int} *in vivo* also has to be predicted from CL_{int} *in vitro*. CL_{int} *in vitro* is measured in µL/(mg*min) or µL/(10^6 cells*min) while CL_{int} *in vivo* is measured in ml/(min*kg body weight) so scaling factors (SF) that relate mg protein or number of cells to g liver, and g liver to kg body weight are used [17]. These scaling factors are different for different species [18]. However, when only taking the SF into account CL_{int} *in vivo* is in many cases under-predicted. One method to correct for these systematic under-predictions is the regression line correction method [19].

When validating new metabolic stability assays or when evaluating different prediction models, well known compounds with existing *in vivo* clearance data are often used. Then, it is common to illustrate the prediction capacity by comparing the predicted CL_{int} *in vivo* with measured CL_{int} *in vivo*. CL_{int} *in vivo* cannot be measured directly but is instead calculated by rearrangement of the well-stirred model, or any of the other models [17].

2.4 Assay validation

During and after the development of a new method it is important to assess the quality of the method. The validation requirements differ depending on what type of method it is, its intended use as well as if it is a completely new method or if its a further development of an already existing method [20, 21]. One common parameter to investigate is the variance of the assay. If an experiment is performed on several occasions the inter-assay variance can be assessed and if the same sample is included more than once in an experiment, the intra-assay variance can be assessed. The percent coefficient of variation (CV%), which is the standard deviation divided by the mean times 100, can be used for simple determination of the intra-assay and inter-assay variations [22, 23]. If inter-assay and intra-assay variation is studied simultaneously, inter-assay variation is usually calculated using the mean of the values within the assay [24].

Other methods to determine the assay variance are also used. For example, AstraZeneca developed a tool called the Manhattan Tool, which is a control chart that uses the data from control compounds to identify periods of stability in the assays and to set acceptance criteria. Another tool that was developed at AstraZeneca and is used to monitor the quality of assays is the Minimum Discriminatory Difference or Ratio (MDD/MDR) which is a measurement of variability in the assay that gives an estimation as to whether the difference between two compounds is likely to be a real difference or not. It can be calculated from any set of compounds where multiple measurements exist according to equation 2.5 and 2.6 [25, 26].

$$\begin{aligned}\text{Intra}-\text{site Var(diff)} = 2 * (&\text{Var(date)} + \text{Var(compound by date)} \\ &+ \frac{\text{Var(replicates)}}{\text{R}})\end{aligned} \tag{2.5}$$

$$\text{MDD} = \text{t} * \sqrt{\text{Var(diff)}} \tag{2.6}$$

Where Intra-site Var(diff) is the total variance of the difference between compounds measured at the same site, Var(date) is the variance between dates, Var(compound by date) is the interaction of compound with date, Var(replicates) is the variance of replicates within date, R is the number of replicates and t is the Student's t-value corresponding to the 95% confidence level. When balanced data is available, i.e. the same number of menausrements of each compound on each date, equation 2.7 can be used to calculate MDD [25].

$$\text{MDD} = \text{t} * \sqrt{\frac{\sum_{\text{i}=1}^{\text{nc}}(\text{n}_1 - 1) * \text{var}_\text{i}}{\sum_{\text{i}=1}^{\text{nc}}(\text{n}_1) - \text{nc}}} * \sqrt{2} \tag{2.7}$$

Where n_i is the number of measurements for compound i, var_i is the data variance for for compound i and nc is the number of compounds used to derive the MDD. When the data is unbalanced, Var(diff) is best calculated by fitting a linear mixed model with dates and compound by date interactions fitted as random effects using the restricted maximum likelihood method. If replicates are present, Var(replicates) has to be estimated as well. MDR is calculated in the same way but based on the logarithms of the individual values. When the resulting value then is anti-logged it changes the difference between two values to a ratio [25].

When comparing two different methods that are used for the same measurement, it is in general necessary to know if the methods are equivalent. If the new method is compared with a golden standard the degree of which the new method measures the real value is called the accuracy. However, in method comparison studies the new method is generally compared with an already established method, not a golden standard, and the differences in measurement obtained with the two methods is then called the bias of the new method relative the established method [27].

Many studies determine the degree of agreement between two methods by calculating the correlation coefficient (r) which is a measure of the linear relationship between two sets of data. However, this method is not optimal since it measures correlation, not difference, between the two methods. Methods that are designed to measure the same variable should have good agreement and a high correlation can therefor just be a sign that a widespread sample was chosen. In addition, good correlation does not automatically imply good agreement since methods can have a strong linear relationship even if there is a systematic deviation between them [28]. Instead, it is more accurate to calculate a fold change or to construct a Bland-Altman plot. When determining the average bias the average fold error (AFE) can be calculated according to equation 2.8. However, in some cases it is more relevant to determine

the average absolute fold error (AAFE), see equation 2.9 [17]. The Bland-Altman plot visualize the bias by plotting the difference between the measurements obtained from the two methods vs the mean of the two measurements. The bias is then the overall mean difference obtained with the two methods. The plot also shows upper and lower limits of agreement (LOA) which are confidence limits of the bias [27].

$$\mathrm{AFE} = 10^{\frac{1}{\mathrm{N}} \sum \log\left(\frac{\text{Method A}}{\text{Method B}}\right)} \tag{2.8}$$

$$\mathrm{AAFE} = 10^{\frac{1}{\mathrm{N}} \sum \left|\log\left(\frac{\text{Method A}}{\text{Method B}}\right)\right|} \tag{2.9}$$

Interpretation of the results from a method-comparison study vary depending on the type of assay. This is true for interpretation of the inter-assay and inter-assay variations as well. Criteria cut-offs are set in advance and the new method is accepted or not based on these criteria [27].

3

Methods

Initial miniaturization experiments were performed in this project and at this stage it was not possible to use an automated platform. If the miniaturized assays are implemented in the Wave 1 screening it would however be necessary to transform the protocols to an automated platform, which was considered throughout the project when different equipment was tested and evaluated.

This section begins with a list of all equipment, materials and chemicals used and is followed by descriptions of the used methodologies.

3.1 Equipment and Chemicals

Equipment
Vprep liquid handler with 384 head (Agilent). Multidrop Combi Reagent Dispenser (ThermoFisher Scientific). Certus Flex micro dispenser (fritz Gyger). Echo 555 Liquid Handler (Labcyte). BBD 6220 CO_2 Incubator (Thermo Scientific). PHMP-4 Thermoshaker (Grant-bio). Sigma 6-16K (Sigma Centrifuges). Sorvall Legend T Benchtop Centrifuge (Thermo Scientific). Rotanta 46 RSC Robotic (Hettich). Casy TT cell counter (Roche Innovatis). Waterbath (Grant). Vacuset (Inotech Biosystems). PlateLoc Thermal Plate Sealer (Agilent).

Materials
Echo Qualified 384-Well Polypropylene Microplate 2.0 (Labcyte, PP-0200). Microplate, 384 well, PP, V-bottom, Natural (Greiner bio-one, 781280). AxyMats AM-384-DW-SQ (Axygen, 12527897). 150-donor mixed gender pooled Human Liver Microsomes (Bioreclamation IVT, product no. X008070, Lot QQY). Cryopreserved hepatocytes male Rat Han Wistar (Bioreclamation IVT, Lot XAP). Breathe-Easy Sealing Membrane (Diversified Biotech).

Chemicals
Acetonitrile (ACN) of LC-MS grade (Fisher Chemical, 15384528). Formic acid (FA) of *p.a.* quality (Fisher Chemical, 10375990), L-15 Leibowitz buffer (Gibco, 21083027). 5,5-Diethyl-1,3-diphenyl-2-iminobarbituric acid (no. 39) (Sigma-Aldrich). Dimethyl Sulphoxide (DMSO) (Sigma-Aldrich, D2650). Casyton (Ols Omni Life

Science, 5651808). Potassium dihydrogen phosphate of *p.a.* quality (Sigma-Aldrich, 1048771000). Di-sodium hydrogen phosphate of *p.a.* quality (Sigma-Aldrich, 1065800500). β-Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (β-NADPH) (Sigma Aldrich, N1630).

Stop solution: 0.2 ml 1 mM no.39 stock solution and 8 ml pure FA was added to 1000 ml ACN to get a stop solution of 200 nM no.39 in 8 % FA. The solution was stored at 4°C.

Phosphate buffer: 100 mL of 100 mM Na_2HPO_4 in water and 30 mL 100 mM KH_2PO_4 in water was mixed and pH was set to 7.4.

3.2 Metabolic stability experiments in 384-well format, using human liver microsomes

A protocol to study metabolic stability in 384-well format using human liver microsomes was developed and optimized regarding four main aspects. These four aspects were the dispensing method of the microsomes, incubation volume, assay-ready plates and sample dilution. The optimized 384-well format assay was then used to repeat a Wave 1 screening of 48 compounds.

3.2.1 Preparation of microsomal incubation mixture

One vial containing 500 µL HLM, stored at -80°C, was thawed in a waterbath at 37°C. A 20 mM NADPH solution was prepared by dissolving 10 mg NADPH in 530 µL phosphate buffer (pH= 7.4). The incubation mixture was prepared by mixing 500 µL HLM and 500 µL of the NADPH solution with 11 mL phosphate buffer.

3.2.2 Initial experiments to test the proposed protocol and evaluate dispensing methods

The six quality control compounds Verapamil (AZ-01), Benzydamine (AZ-02), Imipramine (AZ-03), Phenacetin (AZ-04), Metoprolol (AZ-05) and Diclofenac (AZ-06) that are included in the Wave 1 HLM metabolic stability screening every week were chosen for the first experiments in 384-format since they are well known and since they are metabolized by different CYPs.

The six control compounds were diluted to 2 mM in DMSO and 40 µL of each compound was transferred to six different wells on an Echo qualified 384-well plate (Labcyte, PP-0200). This plate was used as source plate in all experiments that tested the six control compounds. An Echo protocol was made using the Echo

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	15	17	18	19	20	21	22	23	24
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14	A15	A16	A17	A18	A19	A20	A21	A22	A23	A24
B	B1	AZ-01	AZ-01	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15	B16	B17	B18	B19	B20	B21	B22	B23	B24
C	C1	AZ-02	AZ-02	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20	C21	C22	C23	C24
D	D1	AZ-03	AZ-03	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14	D15	D16	D17	D18	D19	D20	D21	D22	D23	D24
E	E1	AZ-04	AZ-04	E4	E5	E6	E7	E8	E9	E10	E11	E12	E13	E14	E15	E16	E17	E18	E19	E20	E21	E22	E23	E24
F	F1	AZ-05	AZ-05	F4	F5	F6	F7	F8	F9	F10	F11	F12	F13	F14	F15	F16	F17	F18	F19	F20	F21	F22	F23	F24
G	G1	AZ-06	AZ-06	G4	G5	G6	G7	G8	G9	G10	AZ-01	AZ-01	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	G23	G24
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	AZ-02	AZ-02	H13	H14	H15	H16	H17	H18	H19	H20	H21	H22	H23	H24
I	I1	I2	I3	I4	I5	I6	I7	I8	I9	I10	AZ-03	AZ-03	I13	I14	I15	I16	I17	I18	I19	I20	I21	I22	I23	I24
J	J1	J2	J3	J4	J5	J6	J7	J8	J9	J10	AZ-04	AZ-04	J13	J14	J15	J16	J17	J18	J19	J20	J21	AZ-01	AZ-01	J24
K	K1	K2	K3	K4	K5	K6	K7	K8	K9	K10	AZ-05	AZ-05	K13	K14	K15	K16	K17	K18	K19	K20	K21	AZ-02	AZ-02	K24
L	L1	L2	L3	L4	L5	L6	L7	L8	L9	L10	AZ-06	AZ-06	L13	L14	L15	L16	L17	L18	L19	L20	L21	AZ-03	AZ-03	L24
M	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12	M13	M14	M15	M16	M17	M18	M19	M20	M21	AZ-04	AZ-04	M24
N	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13	N14	N15	N16	N17	N18	N19	N20	N21	AZ-05	AZ-05	N24
O	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10	O11	O12	O13	O14	O15	O16	O17	O18	O19	O20	O21	AZ-06	AZ-06	O24
P	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23	P24

Figure 3.1: Plate layout showing the position of the six control compounds. There are six replicates of each compound.

Reformat software, which was used in the experiments to transfer compounds from the source plate to the assay plates (Greiner bio-one, 781280). The assay plate layout can be seen in Figure 3.1.

5 µL incubation mixture (prepared according to 3.2.1) was dispensed using the Certus microdispenser to each well that was going to be used on seven plates. All assay plates, except the blank plate and the 0 timepoint plate, were preincubated for 5 min in 37°C, 5% CO_2 and 95 %rH. After preincubation, 2.5 nL of 2 mM compounds were transferred from the source plate to the six timepoint plates using the Echo and the protocol described above. Each compound was transferred to six different wells on each timepoint plate to get six replicates. Ice-cold stop solution was added to the 0 timepoint plate and the blank plate directly using the Multidrop dispenser. These were sealed with aluminum using the PlateLoc sealer and put in 4°C. The remaining 5 plates were centrifuged at 300 x g in 30 seconds and placed in the 37°C, 5% CO_2, 95% rH incubator. The plates were incubated for 5, 10, 15, 20 and 30 minutes, respectively, counting from when the compounds were added to the incubation mixture. The reactions were quenched with the stop solution using the Multidrop dispenser, sealed and put in 4°C. All seven plates were thereafter centrifuged at 3000 x g in 4°C during 20 minutes. Compound disappearance was measured with UPLC-MS/MS as described in section 3.4, data processing was done according to section 3.5 and the results were evaluated as described in section 3.5.1.

A second experiment used two sets of assays plates. One set was treated in the same way as in the previous experiment while the microsomes were dispensed to the other set of plates using the Multidrop instead of Certus. A third experiment was performed where one set of reaction plates was used and the microsomes were dispensed with the Multidrop. The other parts of the experiments were performed as in the first experiment explained above.

3.2.3 Further optimization of assay protocol

After the experiments with the six control compounds a set of 21 validation compounds, named in this report with numbers (AZ-01–AZ-21), were tested. Included

in this set was the six control compounds. The other compounds were chosen since there exists *in vivo* data of the compounds' clearance in humans, rats and dogs. In addition, the compounds have different chemical structures and physochemical properties. Diclofenac (AZ-06) appears in the list twice but the two samples are from different batches. The compounds were ordered from compound management and arrived in a 384-well plate, 40 µL 10 mM in DMSO of each. The compounds were transferred to an Echo qualified plate (Labcyte, PP-0200) and diluted to 2 mM in DMSO. This was the source plate that was used in all HLM experiments that tested the validation compounds. An Echo protocol was constructed using the Echo Reformat software and the layout can be seen in Figure 3.2. Every compound was transferred to three wells on the assay plates (Greiner bio-one, 781280) to get three replicates. Seven assay plates were used for the six timepoints and the blank.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	15	17	18	19	20	21	22	23	24
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14	A15	A16	A17	A18	A19	A20	A21	A22	A23	A24
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15	B16	B17	B18	B19	B20	B21	B22	B23	B24
C	C1	AZ-07	AZ-06	C4	C5	C6	C7	C8	C9	C10	AZ-07	AZ-06	C13	C14	C15	C16	C17	C18	C19	C20	C21	AZ-07	AZ-06	C24
D	D1	AZ-08	AZ-15	D4	D5	D6	D7	D8	D9	D10	AZ-08	AZ-15	D13	D14	D15	D16	D17	D18	D19	D20	D21	AZ-08	AZ-15	D24
E	E1	AZ-09	AZ-06	E4	E5	E6	E7	E8	E9	E10	AZ-09	AZ-06	E13	E14	E15	E16	E17	E18	E19	E20	E21	AZ-09	AZ-06	E24
F	F1	AZ-10	AZ-16	F4	F5	F6	F7	F8	F9	F10	AZ-10	AZ-16	F13	F14	F15	F16	F17	F18	F19	F20	F21	AZ-10	AZ-16	F24
G	G1	AZ-04	AZ-01	G4	G5	G6	G7	G8	G9	G10	AZ-04	AZ-01	G13	G14	G15	G16	G17	G18	G19	G20	G21	AZ-04	AZ-01	G24
H	H1	AZ-11	AZ-17	H4	H5	H6	H7	H8	H9	H10	AZ-11	AZ-17	H13	H14	H15	H16	H17	H18	H19	H20	H21	AZ-11	AZ-17	H24
I	I1	AZ-12	AZ-18	I4	I5	I6	I7	I8	I9	I10	AZ-12	AZ-18	I13	I14	I15	I16	I17	I18	I19	I20	I21	AZ-12	AZ-18	I24
J	J1	AZ-13	AZ-05	J4	J5	J6	J7	J8	J9	J10	AZ-13	AZ-05	J13	J14	J15	J16	J17	J18	J19	J20	J21	AZ-13	AZ-05	J24
K	K1	AZ-14	AZ-19	K4	K5	K6	K7	K8	K9	K10	AZ-14	AZ-19	K13	K14	K15	K16	K17	K18	K19	K20	K21	AZ-14	AZ-19	K24
L	L1	AZ-02	AZ-20	L4	L5	L6	L7	L8	L9	L10	AZ-02	AZ-20	L13	L14	L15	L16	L17	L18	L19	L20	L21	AZ-02	AZ-20	L24
M	M1	AZ-03	AZ-21	M4	M5	M6	M7	M8	M9	M10	AZ-03	AZ-21	M13	M14	M15	M16	M17	M18	M19	M20	M21	AZ-03	AZ-21	M24
N	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13	N14	N15	N16	N17	N18	N19	N20	N21	N22	N23	N24
O	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10	O11	O12	O13	O14	O15	O16	O17	O18	O19	O20	O21	O22	O23	O24
P	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23	P24

Figure 3.2: Plate layout showing the position of the 21 validation compounds in three replicates. The six control compounds are in grey.

The initial experimental protocol was the same which had been used in the last experiment testing the control compounds (see section 3.2.2). The experiment was repeated, varying different parameters to optimize the protocol. Both Certus and Multidrop was used to dispense the microsomes. In some experiments, 5 nL DMSO was added to the wells in the blank plate and the blank plate was also preincubated like the other plates. It was also tested to use 10 µL incubation mixture and 5 nL compound in every reaction well, instead of 5 µL incubation mixture and 2.5 nL compound which had been used previously. To avoid evaporation during the analysis, it was tested to dilute the samples 1:3 with water before analysis. To do this, a protocol was written on the Vprep to transfer 20 µL of the samples to other plates (Greiner bio-one, 781280) containing 40 µL water in each well.

When compound management implement the use of acoustic tubes it will be possible to order so called assay-ready plates. Assay-ready plates are in this context plates that arrive with the right amount of compound in the right wells and the compounds therefore do not have to be transferred from the compound plates to the assay plates which is the case today. The use of assay-ready plates was simulated in this project by transferring the compounds to the assay plates before dispensing the microsomes, using the same Echo 555 protocol as before. The incubation mixture was thereafter

prepared and preincubated in a 15 mL vial in 37°C for 15 minutes. During this time, stop solution was dispensed to the blank plate and the 0 timepoint plate. Stop solution was added to the 0 timepoint plate before the incubation mixture to get a "true" zero timepoint. The incubation mixture was then added using Multidrop and the rest of the experiment was performed as before, including the dilution step at the end.

3.2.4 Screening of 48 Wave 1 compounds

A set of 48 compounds that recently had been screened in the Wave 1 HLM metabolic stability assay was chosen for analysis in the newly developed 384-well format assay. That weeks' compounds were chosen because of the low number of compounds, which would simplify the pooling. When $\leq$ 96 compounds are screened in the existing assay only one incubation plate is used and only two compounds are pooled together before analysis.

The 48 compounds were ordered from compound management and arrived in column one, two and three on an Echo qualified 384-well plate (Labcyte, PP-0200) 20 µL 2mM in DMSO of each. An Echo protocol that would transfer 5 nL of the compounds to the same positions on another plate was constructed. The incubation mixture was prepared according to section 3.2.1 and the experiment was performed as before, with 10 µL incubation mixture dispensed with Multidrop, pre-incubation of all plates, 5 nL compounds in the timepoint plates and 5 nL DMSO in the blank plate. In addition, the compounds were pooled during the dilution step. Plates were prepared with 40 µL water and 10 µL of two different compounds (from the same timepoint) were added to the same well. This was performed manually and due to the pipetting errors the resulting CL_{int} values were calculated using the ratio between the peak area of analytes and peak area of the volume marker. No technical or biological replicates were performed, as this is how the assay is run in the Wave 1 screening. The bias between the two methods was calculated as fold change and was illustrated with a Bland-Altman plot.

3.3 Metabolic stability experiments i 384-well format, using rat hepatocytes

A set of 22 validation compounds was used in the experiments to develop an assay to study metabolic stability i 384-well format using cryopreserved rat hepatocytes. The metabolic stability experiments are described in this section, as well as an experiment that examined cell viability after dispensing with Multidrop.

3.3.1 Preparation of incubation mixture with hepatocytes

Vials containing Rat Han Wistar cryopreserved hepatocytes (7 million cells/mL), stored at -150°C, were thawed in a 37°C water bath and transferred to a Falcon tube

containing 45 mL Leibovitz L-15 buffer. The tube was centrifuged at 50 x g for three minutes at room temperature and the supernatant was discarded using the Vacuset. This washing step was repeated once. The pellet was then resuspended in Leibovitz buffer to get approximately 3 million cells/mL. 25 µL of the suspension was added to 10 mL Casyton and the cell concentration and viability was measured using the CASY Innovatis cell counter. The suspension was then diluted with Leibovitz buffer to get a concentration of 1 million cells/mL.

3.3.2 Development of experimental protocol

The set with validation compounds included the same compounds as the HLM validation set, except the control compounds which were the controls that are used in the weekly Wave 1 metabolic stability screening with hepatocytes. These control compounds are Verapamil (AZ-01), Terfenadine (AZ-22), Indapamide (AZ-23), Dofetilide (AZ-24), Bosentan (AZ-25) and Lorazepam (AZ-26). The compounds were ordered from compound management and arrived in a 384-well Echo Qualified plate (Labcyte, PP-0200), 40 µL 10 mM in DMSO of each. The compounds were diluted to 2 mM in DMSO. This plate was used as source plate in all experiments using hepatocytes. An Echo protocol was constructed using the Echo Reformat software and the layout can be seen in Figure 3.3. Every compound was transferred to three wells on the assay plates (Greiner bio-one, 781280) to get three replicates. Eight assay plates were used for the seven timepoints and the blank.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	15	17	18	19	20	21	22	23	24
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	A14	A15	A16	A17	A18	A19	A20	A21	A22	A23	A24
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15	B16	B17	B18	B19	B20	B21	B22	B23	B24
C	C1	**AZ-01**	**AZ-09**	C4	C5	C6	C7	C8	C9	C10	**AZ-01**	**AZ-09**	C13	C14	C15	C16	C17	C18	C19	C20	C21	**AZ-01**	**AZ-09**	C24
D	D1	**AZ-22**	**AZ-08**	D4	D5	D6	D7	D8	D9	D10	**AZ-22**	**AZ-08**	D13	D14	D15	D16	D17	D18	D19	D20	D21	**AZ-22**	**AZ-08**	D24
E	E1	**AZ-23**	**AZ-06**	E4	E5	E6	E7	E8	E9	E10	**AZ-23**	**AZ-06**	E13	E14	E15	E16	E17	E18	E19	E20	E21	**AZ-23**	**AZ-06**	E24
F	F1	**AZ-24**	**AZ-19**	F4	F5	F6	F7	F8	F9	F10	**AZ-24**	**AZ-19**	F13	F14	F15	F16	F17	F18	F19	F20	F21	**AZ-24**	**AZ-19**	F24
G	G1	**AZ-25**	**AZ-21**	G4	G5	G6	G7	G8	G9	G10	**AZ-25**	**AZ-21**	G13	G14	G15	G16	G17	G18	G19	G20	G21	**AZ-25**	**AZ-21**	G24
H	H1	**AZ-15**	**AZ-12**	H4	H5	H6	H7	H8	H9	H10	**AZ-15**	**AZ-12**	H13	H14	H15	H16	H17	H18	H19	H20	H21	**AZ-15**	**AZ-12**	H24
I	I1	**AZ-10**	**AZ-17**	I4	I5	I6	I7	I8	I9	I10	**AZ-10**	**AZ-17**	I13	I14	I15	I16	I17	I18	I19	I20	I21	**AZ-10**	**AZ-17**	I24
J	J1	**AZ-13**	**AZ-20**	J4	J5	J6	J7	J8	J9	J10	**AZ-13**	**AZ-20**	J13	J14	J15	J16	J17	J18	J19	J20	J21	**AZ-13**	**AZ-20**	J24
K	K1	**AZ-07**	**AZ-14**	K4	K5	K6	K7	K8	K9	K10	**AZ-07**	**AZ-14**	K13	K14	K15	K16	K17	K18	K19	K20	K21	**AZ-07**	**AZ-14**	K24
L	L1	**AZ-18**	**AZ-16**	L4	L5	L6	L7	L8	L9	L10	**AZ-18**	**AZ-16**	L13	L14	L15	L16	L17	L18	L19	L20	L21	**AZ-18**	**AZ-16**	L24
M	M1	**AZ-11**	**AZ-26**	M4	M5	M6	M7	M8	M9	M10	**AZ-11**	**AZ-26**	M13	M14	M15	M16	M17	M18	M19	M20	M21	**AZ-11**	**AZ-26**	M24
N	N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13	N14	N15	N16	N17	N18	N19	N20	N21	N22	N23	N24
O	O1	O2	O3	O4	O5	O6	O7	O8	O9	O10	O11	O12	O13	O14	O15	O16	O17	O18	O19	O20	O21	O22	O23	O24
P	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23	P24

Figure 3.3: Plate layout showing the position of the 22 validation compounds, including the control compounds marked with grey. There are three replicates of each compound.

The vial with the incubation mixture (prepared according to section 3.3.1) was preincubated in 37°C for 20 minutes. During this time, 5 nL of the compounds was transferred from the source plate to seven assay plates and 5 nL DMSO was transferred to the blank plate using the Echo and the protocol described above. 60 µL Stop solution was added to the blank plate and the 0 timepoint plate before 10 µL of the incubation mixture was added to all wells in column 2, 3, 11, 12, 22 and 23 on the eight plates using the Multidrop. The plates were centrifuged at 300 x g

for 30 seconds and then incubated for 5, 15, 20, 30, 45 and 60 minutes, respectively, counting from when the incubation mixture was added to the compounds. The reactions were stopped with 60 µL stop solution and the plates were sealed and centrifuged at 3000 x g in 4°C during 20 minutes. The samples were then diluted 1:3 in water using the Vprep protocol described in Section 3.2.3. The plates were sealed with AxyMats and compound disappearance was measured with UPLC-MS/MS according to 3.4. Data processing was done according to 3.5 and the results were evaluated according to section 3.5.1. This experiment was performed twice.

Another experiment was conducted where the incubation step was performed on a bench top incubator with 500 RPM shaking. During the incubation, the plates were sealed with Breathe-Easy membranes to reduce evaporation. The other steps in the experiment were the same as in earlier experiments.

3.3.3 Viability experiment

To investigate if the Multidrop affected cell viability, a test comparing the cell viability after dispense with the Multidrop and with pipette was conducted. The incubation mixture was prepared as described in section 3.3.1 but the cell count and viability was measured again after the cell concentration had been diluted to 1 million cell/mL. 40 µL of the incubation mixture was pipetted to each well on three columns on a 384-well plate (Greiner bio-one, 781280) and 40 µL was added to the wells on three other columns using Multidrop. The total cell count and viability was measured in eight wells, four of each dispensing method, using the CASY Innovatis cell counter. The plate was incubated for two hours at 37°C, 5% CO_2 and 95%rH. After one hour and two hours, the cell count and viability was measured in eight wells again.

3.4 Analytical method

All metabolic stability samples containing both the compounds and the volume marker (no.39) were semi-quantified using ultra performance liquid chromatography (Waters Acquity) connected to a triple quadrupole tandem mass spectrometer (Waters Acquity XEVO TQS) using electrospray ionization and multiple reaction monitoring. Separation was performed on an Acquity UPLC HSS T3 1.8 µm 2.1x30 mm column. Column temperature was set to 40°C and sample temperature to 10°C. The flow rate was 1.0 mL/min with the gradient profile seen in Figure 3.4. Eluent A= 0.1% formic acid in water and eluent B=0.1% formic acid in acetonitrile.

The MRM tranistions for each compound were optimized automatically using the Masslynx software QuanOptimize. This was done by first injecting the compounds directly on the mass spectrometer, varying the collision energy and choosing the setting that gave the highest intensity of a daughter ion. The compounds were then injected on the column to determine the expected retention time and the response.

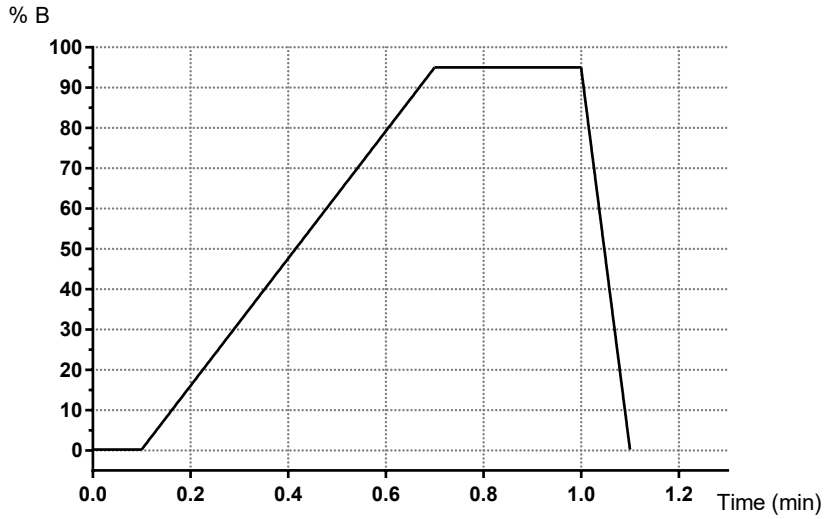


Figure 3.4: Gradient profile of chromatographic method. The Y axis represents the percentage of eluent B in the gradient.

3.5 Data processing

The chromatographic peaks were integrated using the Target Lynx software (Waters) and all peaks were inspected to identify possible abnormalities regarding retention time and peak shape. The data was then imported in Genedata Screener for calculations. The software calculates the compounds' $t_{1/2}$ values and CL_{int} values by plotting the natural logarithm of the peak areas vs the time, constructing a regression line and using the slope of the line to calculate $t_{1/2}$ according to equation 2.1. CL_{int} of HLM experiments was calculated according to equation 3.1 and CL_{int} of experiments with hepatocytes was calculated according to equation 3.2.

$$CL_{int}\ (\mu L/(mg * min)) = \frac{\ln 2 * 1000}{t_{1/2}\ (min)\ * C_{protein}\ (mg/mL)} \tag{3.1}$$

$$CL_{int}\ (\mu L/(10^6 cells * min)) = \frac{\ln 2 * 1000}{t_{1/2}\ (min)\ * C_{protein}\ (10^6 cells/mL)} \tag{3.2}$$

3.5.1 Acceptance criteria and statistical evaluation

The statistical analysis of the depletion curves was also performed using Genedata Screener. If the slopes significantly deviated from zero, a t-value for every regression line was calculated according to equation 3.3 to validate the slopes.

$$t = \frac{r}{\sqrt{(1 - r^2)(n - 2)}} \tag{3.3}$$

Where n is the number of data points and r is the correlation coefficient, which in this case is a measurement of the linear correlation between ln peak area and time. The critical t-value (t_α) limit was calculated as TINV(α; n-2), where α was set to 0.05. A significant correlation between concentration and time was obtained when the t-value of the regression line was higher than t_α.

The CV of the volume marker was calculated for every data set and should not exceed 10%. If it could be assessed that a higher CV was related to anything that affected the compound in the same way, for example injection volume, the ratio between the peak area of the analyte and the peak area of the volume marker could be used for calculating CL_{int}.

In the Wave 1 metabolic stability screenings CL_{int} values below 3 are reported as < 3 and CL_{int} values above 300 are reported as >300. These limits were applied in this project as well. For calculations that included the CL_{int} values, $CL_{int} < 3$ was set to 3 and $CL_{int} > 300$ was set to 300.

Intra-assay and inter-assay variability was determined by calculating CV%. Assay variance was also calculated for the optimized HLM metabolic stability assay by calculating MDD and MDR with the use of an AstraZeneca tool based on equation 2.5 and 2.6. Fold change was calculated to determine bias between the two method formats and AFE and AAFE between the methods were calculated according to equation 2.8 and 2.9. When the compound set with 48 compounds was analyzed, the fold change calculations were complemented with a Bland-Altman plot to illustrate the bias.

3.6 *In vivo* prediction

To determine how well the *in vitro* data from the developed assay aligned with *in vivo* data, the IVIVE model developed by an IVIVE group at AstraZeneca was applied. The correction method of this model was changed in 2016, from using the regression line correction method to using a correction factor (CF) of x 3.

Existing *in vivo* clearance data was used to calculate the measured CL_{int} *in vivo* using the well-stirred model and considering protein binding in blood, see equation 3.4. The results obtained in this project were used to calculate the predicted CL_{int} *in vivo*, taking into account scaling factors, fu_{inc} and the correction factor, see equation 3.5.

$$\text{Measured } CL_{int} \textit{ in vivo} = \frac{CL * Q_h}{(CL - Q_h) * fu_{bl}} \tag{3.4}$$

$$\text{Predicted } CL_{int} \textit{ in vivo} = \frac{CL_{int} \textit{ in vitro} * SF * CF}{fu_{inc}} \tag{3.5}$$

fu_{bl} was calculated using equation 2.4. fu_{pl}, C_{bl}, C_{pl}, Q_h, fu_{inc} and SF data was obtained from an AstraZeneca database. Log Measured CL_{int} *in vivo* was plotted vs log Predicted CL_{int} *in vivo* and the fold under predictions of the CL_{int} values were calculated.

4

Results and Discussion

4.1 Automatisation of metabolic stability assays

Since the Wave 1 assays are used to screen up to several hundred compounds per week it is necessary that the assays are automated. The Hamilton Microlab Star robot is used today but if it should be used for the miniaturized metabolic stability assays a 384 head and a 384-well plate holder would have to be purchased. In addition, evaporation during the incubation on the Hamilton robot might be a problem considering the small volumes. Instead, a CoLab Flex cart from HighRes Biosolutions that was installed in the end of May 2018 might be a better automation solution. This robot incorporates an Echo 555, Multidrop, Bravo liquid handler with 384 head, PlateLoc sealer, humidity incubator and a centrifuge. However it is not possible to shake the plates during the incubation on the CoLab robot. These qualities and limitations were considered throughout the project when the assays were developed and evaluated. In addition, the incubation mixtures are in the existing assays pipetted manually to the 96-well incubation plates but this was not considered to be a suitable way to dispense the incubation mixtures to 384-well plates. Therefore, other equipment replacing pipetting was used when developing the new assays and since the CoLab Flex cart incorporates a Multidrop this equipment was prioritized in the project.

4.2 Metabolic stability experiments in 384-well format, using human liver microsomes

The results from the experiments that were performed to miniaturize the metabolic stability assay using human liver microsomes are summarized and evaluated in this section.

4.2.1 Optimization of the assay protocol

The initial experimental protocol was varied regarding four main aspects to optimize the assay. These four aspects were the method to dispense the microsomal mixture, the use of assay-ready plates, the incubation volume and sample dilution.

4.2.1.1 Evaluation of two different dispensing methods

Two different dispensing equipment for dispensing the incubation mixture, Certus and Multidrop, were compared both in the initial experiments testing the six quality controls compounds and in the later experiments testing the 21 validation compounds. Table 4.1 lists the control compounds with mean CL_{int} values calculated from the 10 most recent screenings of the compounds in the Wave 1 assay as well as which phase I enzyme(s) that is predominant in their metabolism.

Table 4.1: The six control compounds from the Wave 1 HLM screening.

Compound (Cmp no.)	**CL_{int}[1] ± SD (µL/(mg*min))**	**Enzyme**
Verapamil (AZ-01)	266.9 ± 30.5	CYP3A4
Benzydamine (AZ-02)	23.14 ± 8.6	FMO
Imipramine (AZ-03)	16.5 ± 4.7	CYP2C19, 2D6, 3A4, 1A2
Phenacetin (AZ-04)	23.03 ± 2.9	CYP1A2
Metoprolol (AZ-05)	4.66 ± 3.1	CYP2D6
Diclofenac (AZ-06)	289.9 ± 14.8	CYP2C9

[1] Mean of 10 most recent measurements in Wave 1 screening. 96-well format.

Table 4.2 lists the results from three initial experiments that tested the control compounds in 384-well format. Certus was used in Experiment A and for one set of the reactions in Experiment B (B1). Multidrop was used in Experiment C and for one set of reactions in Experiment B (B2). Six technical replicates were used in every experiment. AZ-02 was excluded in the second experiment due to measurement errors.

The CL_{int} values obtained when dispensing the incubation mixture with Certus was similar to the values obtained when dispensing with Multidrop. There was a large difference between Experiment A for AZ-01 and AZ-06 and the other experiments. However, this difference was not seen in Experiment B when the two dispensing methods were tested simultaneously and it could therefore be concluded that the initial differences did not occur due to the dispensing methods. Figure 4.1 shows the plotted ln peak areas with regression lines for all replicates in Experiment B. It can be seen that the replicates that were dispensed with Certus have higher response for AZ-03, AZ-04 and AZ-05. However, the differences were small and since only the slope is used to calculate CL_{int} this difference could be neglected.

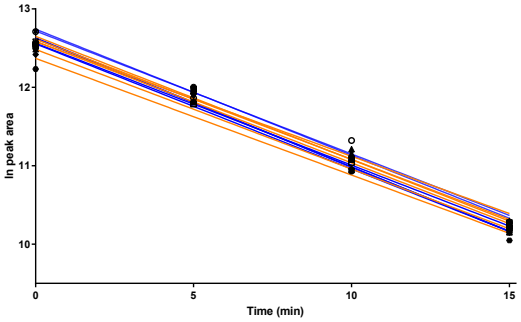
These results were confirmed when the 21 validation compounds were tested. Table 4.4 shows the results from three experiments testing the validation compounds, where the incubation mixture was dispensed with Certus in Experiment E and with Multidrop in Experiment D and F.

Table 4.2: Data from the three metabolic stability experiments with microsomes testing the control compounds. The Average CL_{int} (µL/(mg*min)) values are calculated from n technical replicates. Experiment A and B1 used Certus for dispensing microsomes while experiment B2 and C used Multidrop.

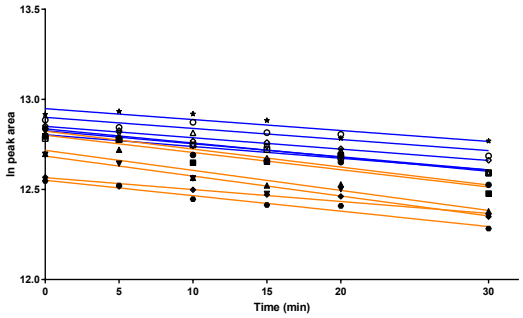
	Experiment A				Experiment B1			
	Average CL_{int}	n	SD	CV	**Average CL_{int}**	n	SD	CV
AZ-01	**267.2**	6	15.8	5.9	**160.3**	6	3.9	2.4
AZ-02	**17.8**	6	1.4	7.7				
AZ-03	**11.9**	6	1.6	13.7	**6.7**	6	0.7	10.6
AZ-04	**10.8**	5	0.7	6.9	**8.5**	5	2.3	27.1
AZ-05	**3.8**	6	1.7	46.1	**3.0**	6	0.0	0.0
AZ-06	**150.0**	6	14.2	9.5	**89.0**	6	3.9	4.4

	Experiment B2				Experiment C			
	Average CL_{int}	n	SD	CV	**Average CL_{int}**	n	SD	CV
AZ-01	**152.2**	6	4.0	2.6	**182.7**	6	10.9	6.0
AZ-02					**20.6**	6	2.9	14.2
AZ-03	**9.5**	6	1.7	17.9	**16.1**	6	2.8	17.3
AZ-04	**10.5**	6	1.9	18.3	**15.9**	6	3.6	22.5
AZ-05	**3.7**	6	1.6	42.1	**3.0**	6	0.0	0.0
AZ-06	**98.7**	6	4.5	4.6	**51.1**	6	8.3	16.2

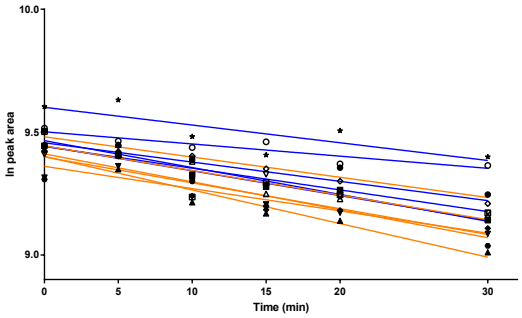
(a) Verapamil (AZ-01)



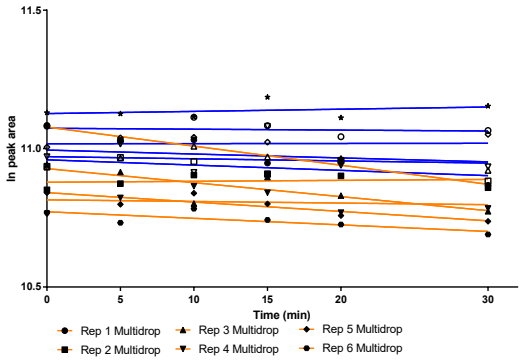
(b) Imipramine (AZ-03)



(c) Phenacetin (AZ-04)



(d) Metoprolol (AZ-05)



(e) Diclofenac (AZ-06)

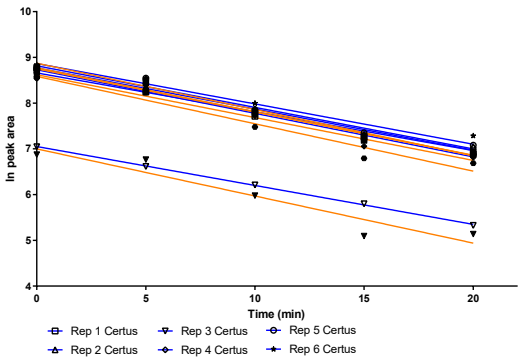


Figure 4.1: Depletion curves of the six control compounds from Experiment B. The replicates that used Certus for dispensing the microsomes are blue and the replicates that used Multidrop are orange. e) The lower responses of two Diclofenac replicates are due to lower injection volumes on the UPLC-MS/MS.

4.2.1.2 Evaluation of different incubation volumes and sample dilution

The initial experimental protocol that was tested used 5 µL incubation mixture and 2.5 nL 2 mM compound per reaction. The CV% of the volume marker within most sample groups of these experiments was more than 10 %, which was the decided acceptable limit. The response of the volume marker was generally higher in the blank samples than in the different timepoint sampels. A possible explanation to the large variance of the volume marker response is that the protein precipitation during the centrifugation step was inadequate and that some constituent of the matrix affected the response of the volume marker. Still, this data could be used to compare Certus and Mutlidrop since the responses of the analytes were within acceptable limits and since the resulting regression lines passed the t-test for all compounds that had slopes that significantly differed from zero, except AZ-04 in Experiment B. The experimental protocol was then changed and it was shown that the CV% of the volume marker decreased when the blank plate was preincubated like the other plates, 5 nL DMSO was added to the blank samples and when the incubation mixture volume and compound volume were increased x 2 (keeping the same compound concentration and microsomal concentration in the incubation). These changes were therefore used for all following experiments.

In every experiment where the analysis on the UPLC-MS/MS was delayed evaporation was an issue despite the AxyMat, probably due to the high concentration of ACN. A dilution step was therefore added in the end of the assay where each sample was diluted 1:3 with water using Vprep. Adding the dilution step successfully decreased evaporation during analysis.

4.2.1.3 Assay-ready plates

The use of assay-ready plates was investigated by dispensing the compounds to the 384-well plates before the microsomes. Two experiments were performed and both times a high difference in response was noted between the technical replicates. In addition, the intra-assay variability of the CL_{int} values was large. Taking the average between the two experiments, 7 of 20 compounds had CV $\geq$ 20% and one compound (AZ-07) could not be measured. The average intra-assay variability for all compounds was 22.6%. The decrease or increase of responses were not random but were seen for all timepoints within different technical replicates for a number of compounds. Since there was one reaction for every timepoint in the 384-well format assay, a systematic error that affected all timepoints of certain compounds within a technical replicate must have occurred. Inter-assay variability was not calculated because only two experiments were performed.

4.2.2 Validation of the assay protocol

Intra-assay and inter-assay variability was determined from three experiments following the developed protocol, with three technical replicates within each experiment. A summary of the assay protocols is seen in Table 4.3. The experiments were

not identical but since it had previously been shown that Certus and Multidrop are equivalent and since the dilution step only affects the evaporation during the analysis and not the metabolic stability the three experiments were treated as three repeats.

Table 4.3: The main changes to the original protocol used in the three experiments that were used for intra-assay and inter-assay variability determination, bias determination and *in vivo* prediction.

	Experiment D	**Experiment E**	**Experiment F**
Incubation volume	10 µL	10 µL	10 µL
Compound volume	5 nL	5 nL	5 nL
Microsomal dispense	Multidrop	Certus	Multidrop
Diluted 1:3 with water	No	No	Yes

Evaporation of samples was an issue in experiment D and E since the samples were not diluted. All technical replicates within these experiments were therefore not included. Table 4.4 shows the resulting CL_{int} values of all compounds calculated as mean of n technical replicates and the standard deviation (SD) and CV% of the technical replicates.

Intra-assay variability was calculated per compound as mean CV% of the technical replicates. 20 of the 21 compounds had intra-assay variability $\leq$ 20 % and the mean intra-assay variability of all compounds was 7.3 %. Inter-assay variability was calculated per compound as CV% of the mean CL_{int} values from the three experiments and the results can be seen in Table A.1 in Appendix A.2. 16 of the 21 compounds had inter-assay variability $\leq$ 20 % and the mean inter-assay variability of all compounds was 12.9 %. Since compounds that have CL_{int} values above 300 or below 3 were reported as >300 and <3, respectively, the variance of these compounds was zero which affects the intra and inter-assay variability. Variance was also determined by calculating MDD and MDR. MDD was 0.23 and MDR was 1.70 when calculated using the mean of the technical replicates. MDD and MDR values from the existing 96-well format HLM assay are shown in Tabel 4.5. The values are calculated every month from CL_{int} data of all compounds that have more than one measurement within the time period. All values are calculated from a time period of three months, giving a two month overlap.

The results were also compared with CL_{int} data obtained when the validation compounds were screened i 96-well format using the same batch of microsomes (QQY). As can be seen in Figure 4.2, CL_{int} values obtained from the 384-well format were in general lower.

The bias was determined by calculating the fold change for every compound and the results are listed in Table A.1 in Appendix A.2. The average fold error (AFE) of all compounds was 1.30 and the average absolute fold error (AAFE) was 1.33. The only compound that had a fold change greater than 2 was Diclofenac (AZ-06). It should be emphasized that the existing 96-well format assay cannot be considered a golden standard, but has deviation from the true value as well. For a new metabolic

Table 4.4: Data from the three metabolic stability experiments with microsomes, testing the validation compounds. The Average CL_{int} (µL/(mg*min)) values are mean from n technical replicates. Experiment E used Certus for dispensing the microsomes while experiment D and F used Multidrop.

	Experiment D				Experiment E				Experiment F			
	Average CL_{int}	n	SD	CV	**Average CL_{int}**	n	SD	CV	**Average CL_{int}**	n	SD	CV
AZ-01	**174.5**	2	10.6	6.1	**160.0**	2	2.8	1.8	**209.0**	3	3.0	1.4
AZ-02	**18.8**	2	1.0	5.3	**16.6**	2	1.3	7.7	**7.8**	3	0.2	2.1
AZ-03	**15.1**	2	1.6	10.3	**10.3**	2	2.1	20.2	**8.9**	3	1.1	12.3
AZ-04	**14.3**	3	1.0	7.3	**9.3**	2	3.3	35.4	**13.8**	3	0.7	4.8
AZ-05	**5.7**	2	1.1	18.7	**3.0**	1			**5.5**	3	0.8	14.5
AZ-06	**106.0**	2	8.5	8.0	**130.5**	2	13.4	10.3	**132.0**	3	6.2	4.7
AZ-06	**108.0**	2	5.7	5.2	**131.5**	2	6.4	4.8	**125.3**	3	4.7	3.8
AZ-07	**221.7**	3	12.2	5.5	**229.0**	2	2.8	1.2				
AZ-08	**3.0**	3	0.0	0.0	**3.0**	2	0.0	0.0	**3.5**	3	0.8	23.3
AZ-09	**3.0**	3	0.0	0.0	**3.0**	2	0.0	0.0	**3.6**	3	1.0	27.1
AZ-10	**3.0**	3	0.0	0.0	**3.0**	2	0.0	0.0	**3.0**	3	0.0	0.0
AZ-11	**3.0**	3	0.0	0.0	**3.0**	1			**3.0**	3	0.0	0.0
AZ-12									**47.9**	3	3.0	6.2
AZ-13	**3.7**	2	1.0	27.8	**3.0**	1			**3.1**	3	0.2	7.4
AZ-14	**3.0**	2	0.0	0.0	**3.0**	2	0.0	0.0	**3.0**	3	0.0	0.0
AZ-15	**7.0**	2	1.2	17.1	**5.4**	2	0.2	4.5	**6.8**	3	0.8	11.3
AZ-16	**4.6**	2	2.3	49.7	**3.0**	1			**3.0**	3	0.0	0.0
AZ-17	**76.5**	2	1.1	1.5	**57.8**	2	6.3	10.9	**72.4**	3	5.5	7.6
AZ-18	**3.0**	2	0.0	0.0	**3.0**	2	0.0	0.0	**3.0**	3	0.0	0.0
AZ-19	**140.5**	2	4.9	3.5	**146.0**	1			**151.0**	3	3.6	2.4
AZ-20	**28.4**	2	0.0	0.0	**26.6**	1			**29.7**	3	0.4	1.5
AZ-21	**27.0**	2	1.3	4.7	**22.3**	1			**30.1**	3	0.2	0.7

Table 4.5: MDD and MDR values for the Wave 1 HLM metabolic stability assay calculated every month 2018 compared to MDD and MDR values from the developed assay.

	Time period	**MDD**	**MDR**
96-well format	Jan - March	0.33	2.13
	Feb - April	0.30	2.01
	March - May	0.33	2.12
	April - June	0.27	1.87
	May - July	0.28	1.91
384-well format		0.23	1.70

stability assay to be accepted it is therefore not only necessary for it to be equivalent to the existing assay, it should also be able to predict *in vivo* clearance sufficiently well. Since the AAFE between the methods was lower than two and since only one compound had fold change >2 the differences were considered small enough for it to be likely that the new method could produce data that would generate sufficiently correct *in vivo* predictions.

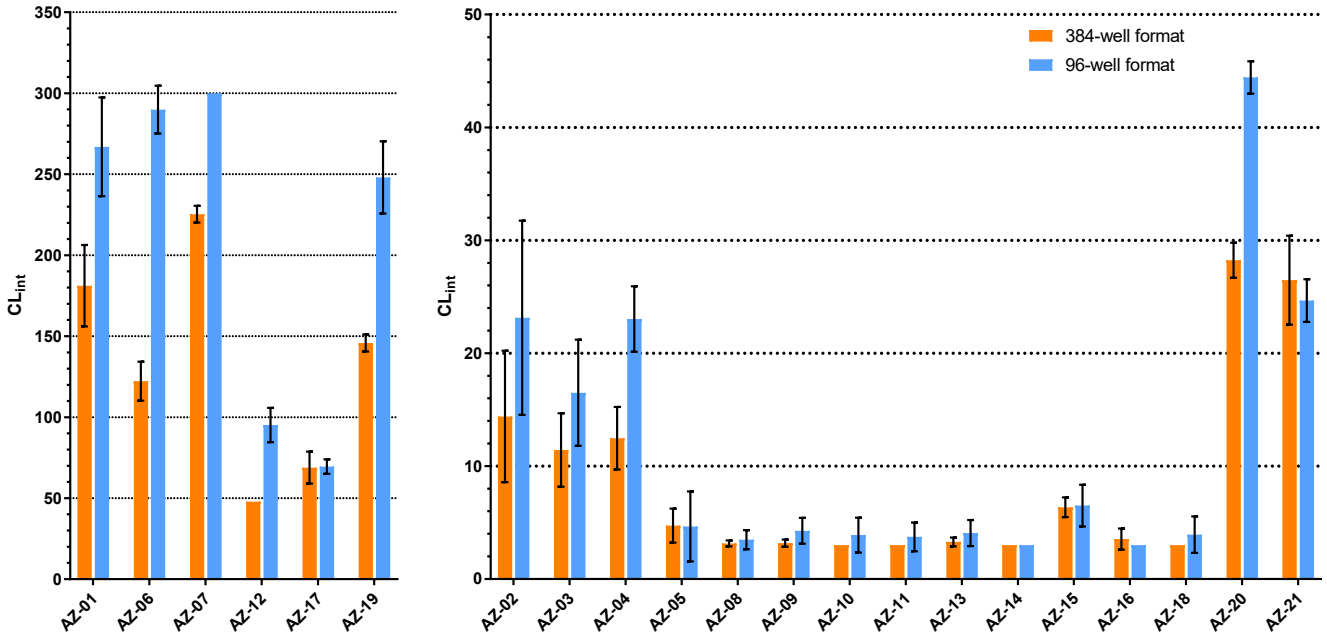


Figure 4.2: Orange bars are mean CL_{int} (µL/(mg*min)) from three metabolic stability experiments in 384-well format. Blue bars are mean CL_{int} (µL/(mg*min)) from three metabolic stability experiments in 96-well format except the control compounds (AZ-01 – AZ-06) which are mean of 10 experiments. Error bars are ±SD.

4.2.3 *In vivo* prediction

To test how well the data from the three experiments D, E and F predicted *in vivo* clearance the prediction model developed by the IVIVE group at AstraZeneca was used. 18 of the validation compounds had the *in vivo* data needed for the prediction. Log Measured CL_{int} *in vivo* was plotted vs log Predicted CL_{int} *in vivo*, see Figure 4.3. Table 4.6 summarizes the results. Diclofenac (AZ-06) and AZ-15 had fold change >5. Diclofenac had the largest fold change also when comparing the 96-well format with the 384-well format while AZ-15 showed similar results in the two assay formats. Studies have previously shown that clearance of Diclofenac commonly is under-predicted from human microsomal CL_{int} data [29]. However, reported CL_{int} *in vitro* in the literature is usually approximately 200 µL/(mg*min) while Diclofenac on average had CL_{int} *in vitro* 122.8 µL/(mg*min) in this project [17, 29].

The prediction plot of a set of 23 validation compounds that were screened in the existing 96-well format HLM assay is visualized in Figure A.1 in Appendix A.2 and a summary of the results are listed in Table 4.6. Comparing the prediction plots of the data obtained in the 384-well format and the data obtained in the 96-well format it is clear that the prediction capacities are similar. The 96-well format has a somewhat higher ratio <2 fold under-prediction (52.2% compared with 33.3%) but the samples tested were not identical.

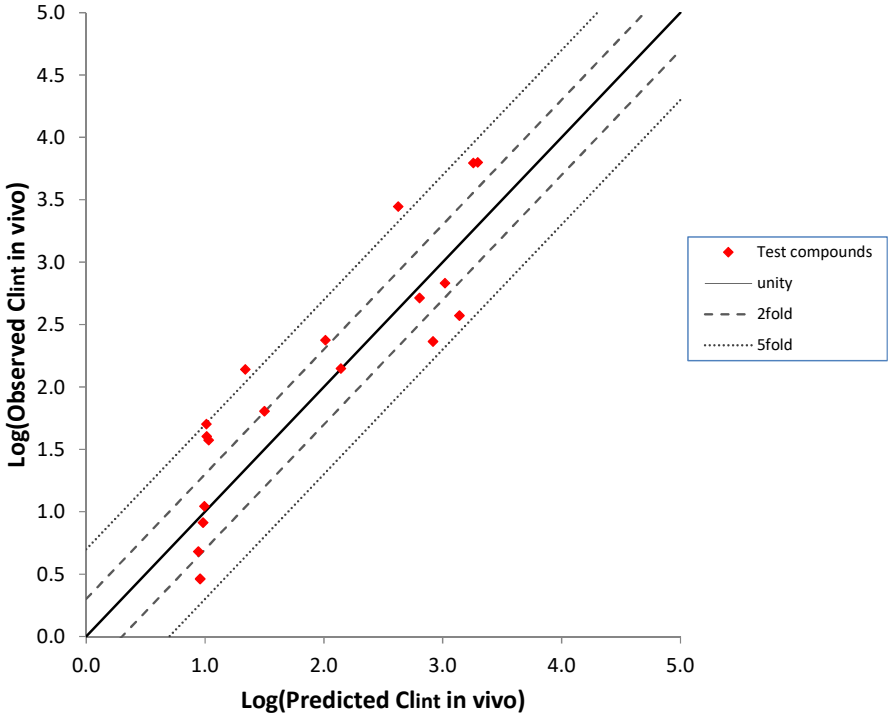


Figure 4.3: *In vivo* prediction of 18 compounds screened in the miniaturized metabolic stability assay using human liver microsomes.

Table 4.6: Fold under-prediction between Predicted CL_{int} *in vivo* and Measured CL_{int} *in vivo* of the 18 compounds screened in 384-well format and the 23 compounds screened in 96-well format.

		<2 fold	2-5 fold	5-10 fold	>10 fold
384-well format	no. cmpds	6	10	2	0
	%	33.3	55.6	11.1	0
96-well format	no. cmpds	12	9	2	0
	%	52.2	39.1	8.7	0

4.2.4 Screening of 48 compounds using the developed assay

One experiment was performed where the developed and validated miniaturized HLM metabolic stability assay was used to screen a set of 48 compounds that recently had been screened in the Wave 1 assay. The resulting CL_{int} values were compared with the results from the screening in 96-well format. A summary of the existing protocol and the developed protocol can be seen in Figure 4.4.

Ten compounds were not detected during the analysis or had abnormal chromatograms and were therefore excluded in the calculations. Two of these compounds failed in the Wave 1 screening as well. One compound was excluded since it did not pass the t-test despite that the depletion curve significantly differed from zero. The CV of the volume marker was outside the normal range for three of the compounds, possibly due to the pipetting during the pooling. The CL_{int} values were therefore calculated based on the ratio between the peak area of the volume marker and the

analyte for all compounds. However, due to the pooling of the samples the pipetting errors could still have affected the calculated CL_{int} values. Of the 37 measured compounds, 4 compounds had fold change >2 and AFE and AAFE were 1.13 and 1.33, respectively.

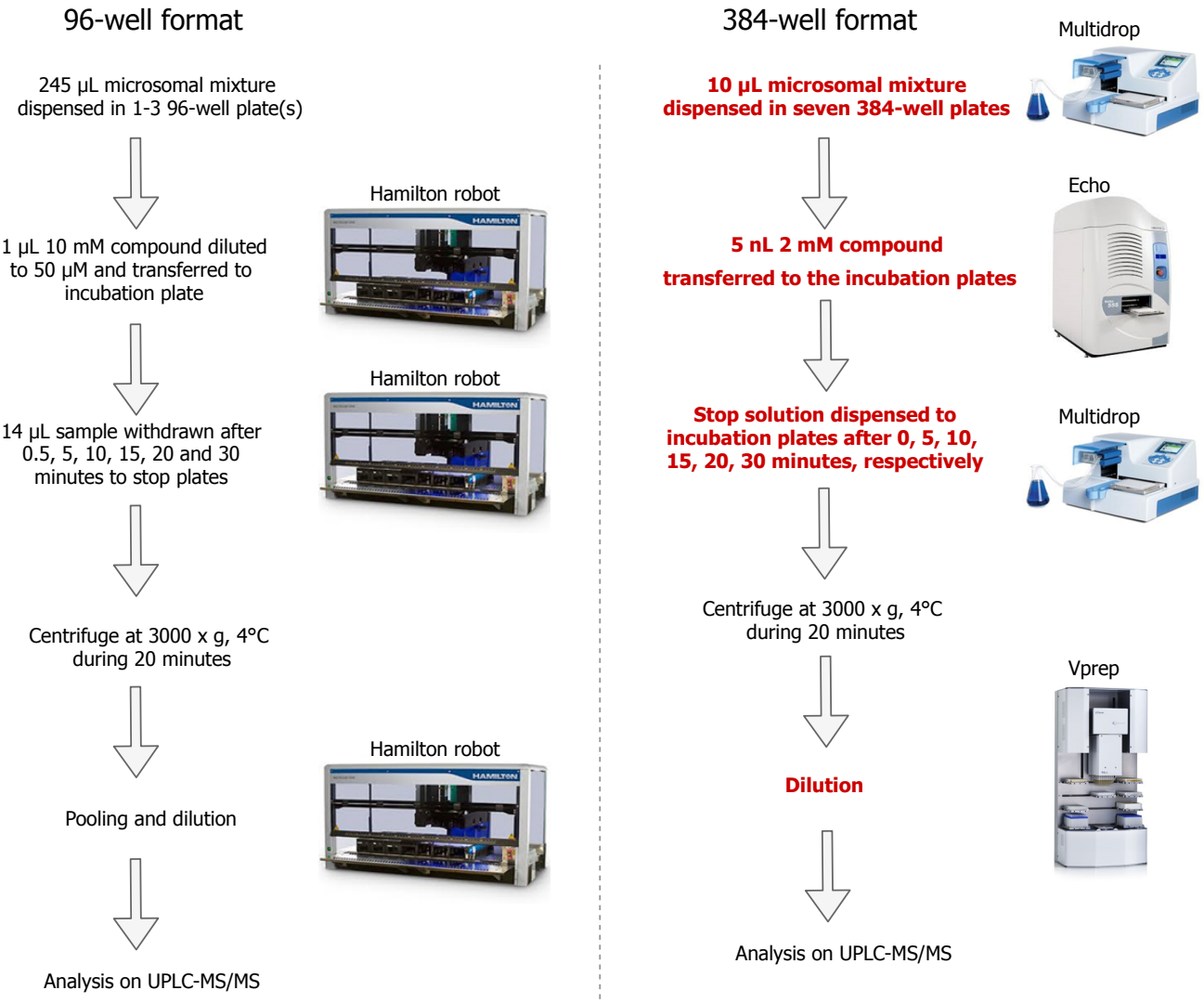


Figure 4.4: Summary of the two different assay formats. The existing assay use one incubation plate and stops the reactions in different stop plates by taking samples from the incubation plate. The miniaturized assay use one incubation plate per timepoint and stops the reactions directly in the incubation plates. The steps of the protocol that were developed in this project are marked in red.

The Bland-Altman plot of the results are visualized in Figure 4.5. Mean difference between the two assays was 9.0, i.e. the CL_{int} values obtained in the 384-well assay were on average 9.0 µL/(mg*min) lower than the values obtained in the 96-well format. The pink lines are the 95% limits around the average and if both lines are on the same side of the zero line, the bias is statistically significant. The lower bias limit was for this data directly under the zero line. However, even if the bias is significant it does not automatically imply that an assay should be rejected. As described before, acceptance criteria should be established for every unique case and assay. The orange lines are the 95% limits of agreement for a future sample, i.e. there is a 95% chance that the difference between future measurements lie within these limits. As seen in the figure, the difference is dependent on the mean and this difference is also significant ($p<0.05$). Diclofenac was the compound with largest difference between CL_{int} in 96-well format and CL_{int} in 384-well format which is in line with previous results.

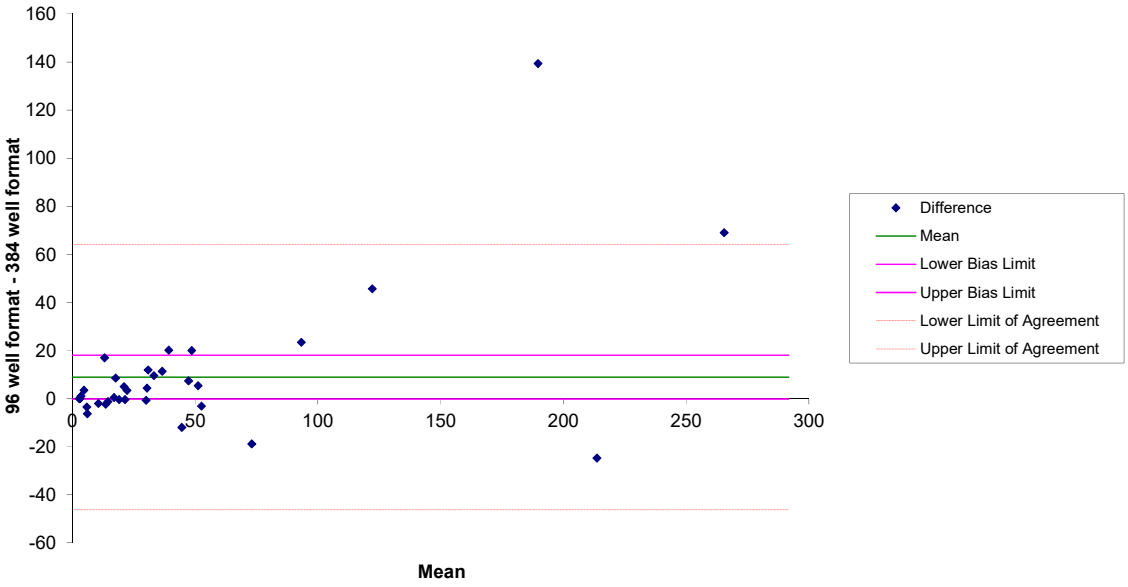


Figure 4.5: Bland-Altman plot of 37 compounds.

4.3 Metabolic stability experiments i 384-well format, using rat hepatocytes

The results from the experiments that were performed to miniaturize the metabolic stability assay using rat hepatocytes are summarized and evaluated in this section.

4.3.1 Optimization and evaluation of the assay protocol

Two initial experiments with three technical replicates of each compound were performed using assay-ready plates and incubation in the 37°C, 5% CO_2 humidity incubator. The resulting CL_{int} values are listed in Table 4.7.

Table 4.7: Data from the three metabolic stability experiments with hepatocytes, testing the validation compounds. The Average CL_{int} (µL/(10^6 cells*min)) values are mean from n technical replicates. Experiment I is here the experiment with shaking during incubation.

	Experiment G				Experiment H				Experiment I			
	Average CL_{int}	n	SD	CV	**Average CL_{int}**	n	SD	CV	**Average CL_{int}**	n	SD	CV
AZ-01	**18.8**	3	1.5	7.9	**18.1**	3	0.3	1.9	**50.4**	3	3.1	6.2
AZ-06	**58.5**	3	4.7	8.8	**40.4**	3	1.7	4.2	**51.1**	3	5.7	11.2
AZ-07												
AZ-08	**4.8**	3	0.3	5.5	**4.4**	3	0.5	12.2	**3.7**	3	0.1	2.0
AZ-09	**60.0**	2	4.9	8.1	**57.5**	3	4.4	7.6	**64.0**	3	8.4	13.2
AZ-10	**5.2**	3	0.5	10.3	**5.0**	3	1.0	20.1	**4.6**	3	0.5	11.8
AZ-11	**8.2**	3	0.9	10.7	**6.2**	3	0.7	10.5	**10.8**	3	0.5	4.8
AZ-12	**49.3**	3	5.7	11.6	**34.8**	3	2.9	8.5				
AZ-13	**9.2**	3	0.3	3.5	**10.6**	3	0.7	6.8	**25.6**	3	0.4	1.6
AZ-14									**9.6**	3	1.1	11.3
AZ-15	**4.1**	3	0.3	6.9	**3.5**	3	0.4	9.9	**9.4**	3	0.5	5.6
AZ-16	**8.6**	3	1.3	15.2	**8.9**	3	0.7	7.6	**15.6**	3	1.0	6.1
AZ-17	**130.7**	3	0.6	0.4	**106.0**	3	4.4	4.1	**108.0**	3	2.6	2.4
AZ-18	**1.0**	3	0.0	0.0	**1.1**	3	0.2	20.8	**1.2**	3	0.3	26.8
AZ-19	**59.5**	3	2.3	3.8	**47.3**	3	2.6	5.5	**101.3**	3	6.5	6.4
AZ-20	**6.9**	3	0.2	2.9	**6.9**	3	0.2	2.6	**10.9**	3	0.1	0.5
AZ-21	**8.1**	3	0.7	8.8	**8.1**	3	0.2	1.7	**20.3**	3	0.3	1.4
AZ-22	**28.6**	3	1.6	5.6	**14.2**	3	1.9	13.6	**45.8**	3	6.5	14.2
AZ-23	**8.5**	3	0.9	10.3	**6.4**	2	1.5	23.4				
AZ-24	**4.4**	3	0.3	6.3	**4.3**	3	1.7	39.5	**8.1**	3	0.5	6.2
AZ-25	**8.9**	3	0.4	5.0	**8.1**	3	0.2	2.7	**14.1**	3	1.1	8.0
AZ-26	**8.0**	3	0.8	9.6	**6.0**	3	0.1	2.2	**14.3**	3	1.6	11.3

19 of the 22 compounds had intra-assay variability ≤ 20 % (two compounds were not measured) and the mean intra-assay variability of all compounds was 8.7 %. Inter-assay variability was not calculated because only two experiments were performed. The obtained CL_{int} values were in general lower than CL_{int} values obtained from

the Wave 1 screening, see Figure 4.6. The fold change between the methods for each compound are listed in Table A.2 in Appendix A.3. 13 of the 20 measured compounds had an average fold change ≥ 2 and AFE and AAFE were 2.19 and 2.52, respectively. The difference between the two assay formats was largest for the high CL_{int} compounds, see Figure 4.6. Since hepatocytes in solution rapidly sediment, it was proposed that the low turnover was obtained due to the absence of shaking during the incubation. If the hepatocytes sink to the bottom, the contact with the compounds decrease and therefore also the biotransformation. To test this hypothesis, an experiment using a benchtop incubator with horizontal shaking was performed. The results were improved but the turnover was still lower than the turnover obtained in the 96-well format assay. AFE decreased to 1.29 and AAFE to 1.62, but 7 of the 19 measured compounds still had an average fold change ≥ 2. Also, the compound that had the largest fold change in the first experiments (AZ-12) could not be detected in the 384-well format assay with shaking which probably affected the AFE and AAFE values. The fact that the miniaturized assay in this project measured metabolic stability during 60 minutes while the existing assay measure during 120 minutes could have affected the bias slightly. However, it was noticed that due to the low incubation volumes and small surface of the wells the mixing of the solution was not fully achieved even with shaking and this was still believed to be the main explanation to the bias. Since the high CL_{int} compounds had a large bias relative the existing method and since the available automated equipment at AstraZeneca did not include any shaker together with incubator, it was decided that no more experiments to miniaturize the assay with hepatocytes should be performed in this project.

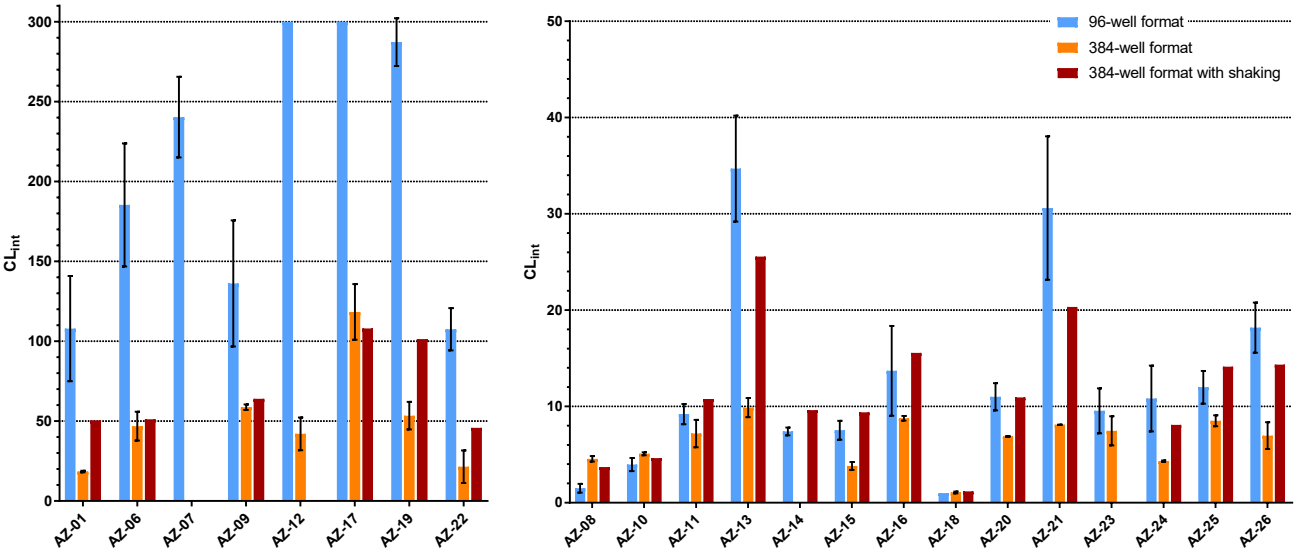


Figure 4.6: Blue bars are mean CL_{int} (µL/(10^6 cells*min)) from four metabolic stability screenings in 96-well format, except the control compounds (AZ-01, AZ-22 – AZ-26) that are mean from 10 experiments. Orange bars are mean CL_{int} (µL/(10^6 cells*min)) in 384-well format, n=2. Red bars are mean CL_{int} (µL/(10^6 cells*min)) in 384-well format where the incubation was performed with shaking at 500 rpm. Error bars are ±SD.

4.3.2 Viability experiment

One experiment to investigate if the Multidrop affected cell viability was performed, where Multidrop was compared with pipetting. Viability was measured three times during two hours in four different wells each. A t-test showed that the difference between Multidrop and pipetting was not significant ($p>0.05$).

Table 4.8: Cell viability after dispensing with Multidrop or pipette. The table shows both total amount of viable cells and percentage viable cells. Average of four measurements per hour.

		0 hour	1 hour	2 hours
Multidrop	Tot	1.26E+06	1.18E+06	1.15E+06
	Viab	1.01E+06	9.96E+05	9.86E+05
	%	**80.1**	**84.3**	**85.7**
Pipette	Tot	1.17E+06	1.14E+06	1.30E+06
	Viab	1.03E+06	1.02E+06	1.14E+06
	%	**87.9**	**89.8**	**87.5**

5

Conclusion

In this study, two metabolic stability assays used for screening of drugs in 96-well format were miniaturized to 384-well format. One major difference between the existing assays and the miniaturized assays was except the format that the reactions in the existing assays take place in one well per compound, from which samples are taken while the developed protocols used one well per timepoint. The resulting protocols were optimized and in the final versions 60 µL and 90 µL incubation mixture was used per compound in the microsome assay (six timepoints) and hepatocyte assay (nine timepoints), respectively. In the existing 96-well format assays, 245 µL incubation mixture is used per compound. Compound usage is in the existing assays 1 µL 10 mM of each compound. The miniaturized microsomal assay uses 30 nL 2 mM of each compound, which is a 167 x decrease compared to the existing assay. The miniaturized assay with hepatocytes uses 45 nL 2 mM of each compound, which is a 111 x decrease. 288 compounds (282 test compounds and six controls) can be screened weekly using the existing assay format. The developed assays have the capacity to screen 384 compounds (378 test compounds and six controls) which is a 34% capacity increase.

The miniaturized microsomal metabolic stability assay produced data with average intra-assay variability 7.3% and inter-assay variability 12.9%. Compared with the existing assay, AFE was 1.30 and AAFE was 1.33. The *in vivo* prediction model showed that the data align well with *in vivo* clearance data. These results lead to the conclusion that the miniaturized metabolic stability assay with microsomes is likely to have equivalent quality as the existing 96-well format assay. To verify this, more experiments should be performed. Because of the large changes to the assay protocol the assay quality has to be assessed over longer time and with more compounds. Except from verifying the quality of the data, the assay also has to be automated before it can replace the existing assay. Since the Multidrop was shown to not affect microsomal activity and since the Bravo can be used instead of the Vprep used in this project the CoLab Flex robot is considered a promising automation platform. The use of assay-ready plates resulted in low quality data in this project. The reason behind these results was not investigated further and therefore a conclusion regarding assay-ready plates will not be drawn at this stage.

The miniaturized metabolic stability assay with hepatocytes produced data with average intra-assay variability 8.7%. Without shaking during the incubation, AFE

was 2.10 and AAFE 2.52 compared to the 96-well format assay and 13 of the 20 compounds had an average fold change >2. Adding shaking during the incubation improved the results but the turnover was still low. Probably the shaking was insufficient for mixing the solution due to the low incubation volume and small surface of the wells compared to the 96-well format. Also the square shape of the wells makes mixing more complicated. In this project it was shown that the Multidrop did not affect cell viability and therefore cell viability was not considered to be the reason behind the low turnover.

The CoLab Flex robot does not have any shaking solution so until another automation platform which can shake the plates during the incubation is available the miniaturized metabolic stability assay with hepatocytes will not be considered for replacing the existing assay. The Hamilton Microlab Star robot can shake plates during incubation but new parts that fit the 384-well format needs to be purchased if the assays should be run on that robot.

6

Future work

It was recognized in this project that miniaturization of the metabolic stability assay using microsomes is likely to be beneficial. To be able to implement the new assay format additional experiments should be performed to asses the quality over time. A template for ordering the compounds in 384-well format would need to be constructed as well as scripts for performing the assays on the CoLab Flex robot, or any other automation platform. The script should include pooling of the compounds since that step is needed to decrease analysis time. To further decrease compound and incubation mixture consumption the reasons behind the issues with the first experiments that used half the amount of compound and incubation mixture could be more thoroughly investigated. In addition, if it is desirable to use assay-ready plates more experiments should be performed to evaluate if a protocol which uses assay-ready plates can be developed to meet the quality criteria. If an automation platform with shaking becomes available it would be beneficial if the hypothesis that lack of mixing was the main reason behind the insufficient results from the assay with hepatocytes was verified.

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A

Appendix

A.1 Wave 1 metabolic stability protocols

This section describes the existing Wave 1 metabolic stability experimental protocols. Preparation of incubation mixtures and the analytical methods are left out since these steps were not changed in this project and has been described earlier in this report.

Metabolic stability assay using human liver microsomes
1. 245 µL incubation mixture is pipetted to each well on the 96-well plates using a multipipette and 20 µL is pipetted to each well on the blank plate if one incubation plate is used, 40 µL if two incubation plates are used and 42 µL if three incubation plates are used.

2. The compound plates containing 1 µL 10 mM of each compound and the incubation plates are put on the Hamilton Microlab Star robot and the script is started.

3. The incubation plates are pre-incubated for 15 minutes, 37°C and 13 Hz and the compound plate is diluted to 50 µM with 50% ACN in water.

4. The reactions are started by adding 5 µL of 50 µM test compound to the incubation plates ending up with a final substrate concentration of 1 µM, protein concentration of 1 mg/mL and 1 mM NADPH. The incubation is continued at 37°C and 13 Hz.

5. 14 µL is withdrawn from each incubation plate after 0.5, 5, 10, 15, 20 and 30 minutes and quenched in 168 µL cold stop solution containing ACN and 19 nM of the volume marker no.39 if three incubation plates are used. If two incubation plates are used, 20 µL is withdrawn from both plates at every timepoint and quenched in 160 µL stop solution. If one incubation plate is used, 20 µL is withdrawn from the incubation plate and quenced in 80 µL stop solution. There is one stop plate per timepoint and compounds from the different incubation plates are pooled together according to Figure 1.2 in the Introduction section.

6. The stop plates are centrifuged for 20 minutes at 4°C and 3000 x g.

7. The stop plates are put on the Hamilton robot again and 35 µL supernatant from two wells are transferred to analysis plates and mixed with 70 µL water (1:4

dilution of the analytes).

Metabolic stability assay using rat hepatocytes

1. 245 µL incubation mixture is pipetted to each well on the 96-well plates using a multipipette and 20 µL is pipetted to each well on the blank plate if one incubation plate is used, 40 µL if two incubation plates are used and 42 µL if three incubation plates are used.

2. The compound plates containing 1 µL 10 mM of each compound and the incubation plates are put on the Hamilton Microlab Star robot and the script is started.

3. The incubation plates are pre-incubated for 15 minutes, 37°C and 13 Hz and the compound plate is diluted to 50 µM with 50% ACN in water.

4. The reactions are started by adding 5 µL of 50 µM test compound to the incubation plates ending up with a final substrate concentration of 1 µM and cell concentration of 10 million cells/mL. The incubation is continued at 37°C and 13 Hz.

5. 14 µL is withdrawn from each incubation plate after 0.5, 5, 15, 30, 45, 60, 80, 100 and 120 minutes and quenched in 168 µL cold stop solution containing ACN, 8% FA and 19 nM no.39 if three incubation plates are used. If one or two incubation plates are used, 112 µL stop solution is used. There is one stop plate per timepoint and compounds from the different incubation plates are pooled together according to Figure 1.2 in the Introduction section.

6. The stop plates are centrifuged for 20 minutes at 4°C and 3000 x g.

7. The stop plates are put on the Hamilton robot again and 35 µL supernatant from two wells are transferred to analysis plates and mixed with 70 µL water (1:4 dilution of the analytes).

A.2 Complementary data - Experiments with microsomes

Table A.1: Data from the three experiments that measured metabolic stability using microsomes. Intra-assay variability calculated per compound as mean of the CV% of the technical replicates from each of the three experiments. Inter-assay variability calculated per compound as CV% of the average CL_{int} from all three experiments. Fold change calculated as ratio between CL_{int} values obtained from the 96-well format assay and CL_{int} values obtained from the 384-well format assay. The two sets of results for AZ-06 were equivalent and therefore pooled together.

	Intra-assay (%)	Inter-assay (%)	Bias (fold change)	
AZ-01	3.1	13.9	1.47	
AZ-02	5.0	40.5	1.61	
AZ-03	14.3	28.5	1.44	
AZ-04	15.8	22.2	1.85	
AZ-05	16.6	31.8	0.98	
AZ-06	6.1	9.9	2.37	
AZ-07	3.4	2.3	1.33	
AZ-08	7.8	8.5	1.11	
AZ-09	9.0	10.1	1.34	
AZ-10	0.0	0.0	1.30	
AZ-11	0.0	0.0	1.24	
AZ-12	6.2		1.99	
AZ-13	17.6	11.9	1.24	
AZ-14	0.0	0.0	1.00	
AZ-15	11.0	13.8	1.02	
AZ-16	24.8	26.5	0.85	
AZ-17	6.7	14.3	1.01	
AZ-18	0.0	0.0	1.00	
AZ-19	3.0	3.6	1.70	
AZ-20	0.7	5.5	1.57	
AZ-21	2.7	14.9	0.93	
Average	**7.3**	**12.9**	**AFE**	1.30
			AAFE	1.33

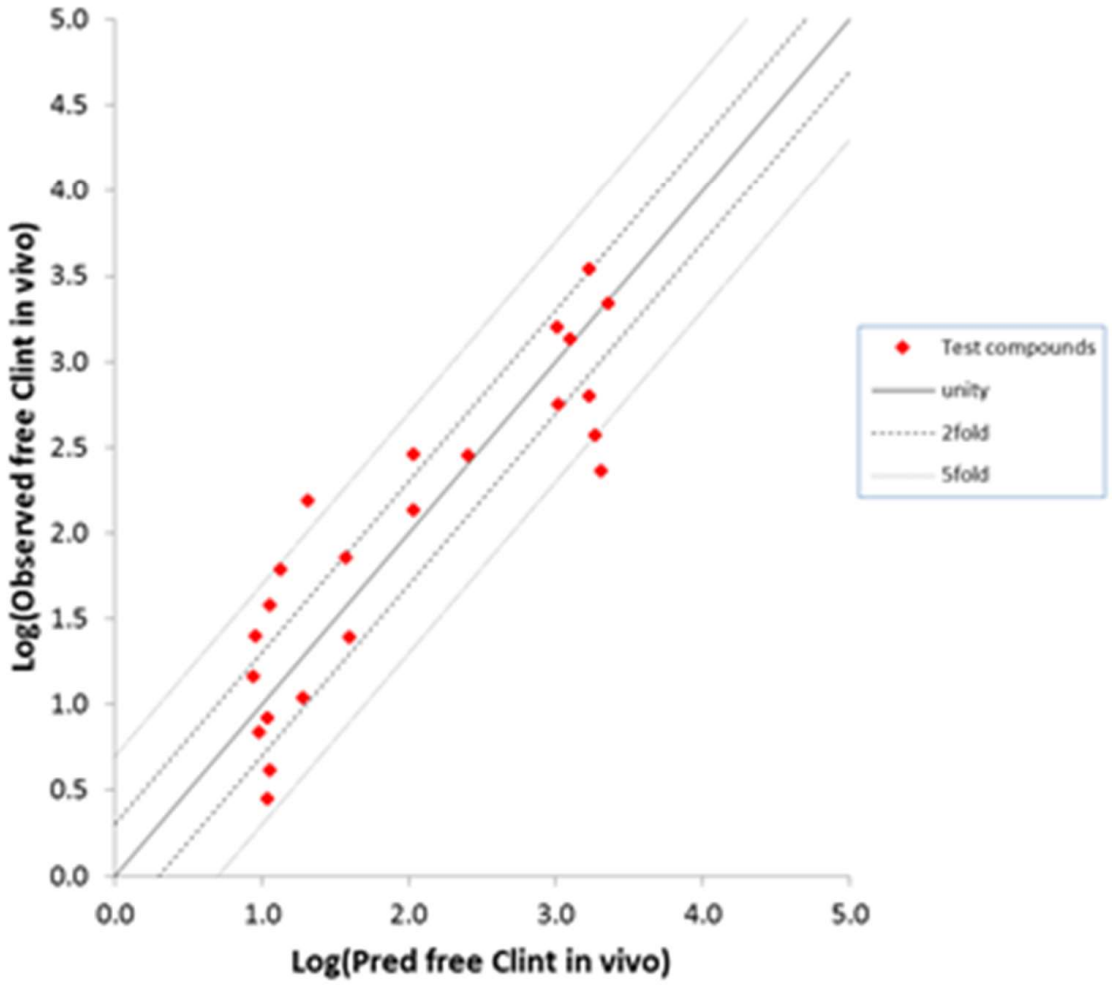

Figure A.1: *In vivo* prediction of 23 validation compounds that were screened in the 96-well format HLM metabolic stability assay.

A.3 Complementary data - Experiments with hepatocytes

Table A.2: Data from the three experiments that measured metabolic stability using hepatocytes. Intra-assay variability calculated per compound as mean of the CV% of the technical replicates from each of experiments. Inter-assay variability calculated per compound as CV% of the average CL_{int} from all three experiments. Fold change calculated as ratio between CL_{int} values obtained from the 96-well format assay and CL_{int} values obtained from the 384-well format assay.

	Intra-assay (%)	Bias $\frac{96-\text{well format}}{384-\text{well format}}$		Bias $\frac{96-\text{well format}}{384-\text{well format with shaking}}$	
AZ-01	4.9	5.8		2.1	
AZ-06	6.5	4.0		3.6	
AZ-07					
AZ-08	8.8	0.3		0.4	
AZ-09	7.8	2.3		2.1	
AZ-10	15.2	0.8		0.9	
AZ-11	10.6	1.3		0.9	
AZ-12	10.0	7.1			
AZ-13	5.2	3.5		1.4	
AZ-14				0.8	
AZ-15	8.4	2.0		0.8	
AZ-16	11.4	1.6		0.9	
AZ-17	2.3	2.5		2.8	
AZ-18	10.4	0.9		0.8	
AZ-19	4.7	5.4		2.8	
AZ-20	2.8	1.6		1.0	
AZ-21	5.3	3.8		1.5	
AZ-22	9.6	5.0		2.3	
AZ-23	16.9	1.3			
AZ-24	22.9	2.5		1.3	
AZ-25	3.9	1.4		0.8	
AZ-26	5.9	2.6		1.3	
Average	**8.7**	**AFE**	2.19	**AFE**	1.29
		AAFE	2.52	**AAFE**	1.62