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Validation of a semi-automated human hepatocyte assay for the determination and prediction of intrinsic clearance in discovery

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Abstract

An automated high throughput human hepatocyte assay has been established with a 96-well format using a Tecan Genesis™ Workstation. Validation of this assay was performed with nine commercially available compounds and an additional 10 Pfizer compounds with varying hepatic extraction ratios (E_H) ranging from 0.02 to ∼1. The incubation conditions in the automated assay are readily and precisely controlled and cell viability of over 80% was achieved in the automated assay further confirming its utility for absorption, distribution, metabolism, and excretion (toxicity) (ADME (T)) screening. The results of the nine commercial compounds correlate with both manually executed ($R^2 = 0.97$) and literature reported experimental results ($R^2 = 0.93$). Overall, measured E_Hs were within two-fold of the literature values for approximately 90% of the 19 compounds tested. Additionally, good inter- and intra-day reproducibility was observed for all the 19 compounds. In conclusion, an automated and robust assay suitable for simultaneously testing up to 48 compounds with multiple time points has been validated. Throughput of 192 compounds per run can be achieved using 384-well plates to meet increasing needs in drug discovery. Currently, this automated assay is used to support early discovery profiling towards lead optimization of various discovery targets/programs.
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Keywords: Human hepatocytes; Automation; Clearance prediction

1. Introduction

New drug candidates for oral delivery need to have desirable pharmacokinetic properties such as a suitable absorption, metabolic clearance and adequate bioavailability. Nearly 40% of drugs in the pipeline failed due to poor biopharmaceutics/pharmacokinetic (PK) properties [1]. One major component of drug disposition that contributes to the success of commercialization of drugs is low human hepatic clearance thus facilitating once-a-day dosing. Cryopreserved human hepatocytes have been demonstrated to be a useful model for the estimation of hepatic extraction ratio (E_H) and hence serve as a powerful in vitro tool for the prediction of this property [2–6]. Since the number of new NCEs to be screened for potential drug candidates has increased due to advances in combinatorial chemistry and other enabling technologies, reliable automated assays for absorption, distribution, metabolism, and excretion (toxicity) (ADME (T)) screening is becoming increasingly necessary to meet the throughput needs and timelines in early discovery.

This work describes an automated, robust human hepatocyte clearance assay for the estimation of intrinsic hepatic clearance in humans. This assay was based on an existing protocol using a manual format [7,8]. Cryopreserved human hepatocytes have been reported to retain most of the phase I and some phase II metabolic activities, and therefore, more closely resemble in vivo metabolism in contrast to sub cellular fractions such as microsomes and liver S-9 fractions [2–6,9]. Additionally cryopreserved hepato-

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Table 1
List of model compounds (literature and Pfizer in-house compounds) chosen for assay validation studies

Literature compound	Human in vivo mean (E_H)	Major metabolizing phases I and II enzymes	Literature reference
Tolbutamide	0.02	CYP2C9	[15]
Triazolam	0.23	CYP3A	[16]
Zolpidem	0.28	CYP3A4	[17]
Nortriptyline	0.35	CYP2D6	[18]
Chlorpromazine	0.55	CYP2D6, CYP1A2, UGT	[19]
Metoprolol	0.68	CYP2D6	[20]
Propranolol	0.70	CYP2D6, CYP1A2, UGT	[21]
Lidocaine	0.75	CYP3A	[22]
Propafenone	0.95	CYP2D6	[23]

Pfizer compound	Estimated human in vivo mean[a] (E_H)
PF1	0.52
PF2	1.00
PF3	0.73
PF4	0.80
PF5	0.06
PF6	0.35
PF7	0.02
PF8	0.16
PF9	0.22
PF10	0.49

[a] Based on Pfizer internal research reports.

cytes are now readily commercially available from many venders.

Automation of cell based assays are generally challenging as vigorous and repeated pipetting can shear the cell membranes causing low and variable cell viability. In developing this automated assay, adequate care was exercised to optimize the liquid handling on TECAN to allow for hepatocyte pipetting in order to maintain the viability of the cells while at the same time improving throughput and robustness.

Nine literature/model compounds (Table 1, Tolbutamide, Triazolam, Zolpidem, Nortriptyline, Chlorpromazine, Metoprolol, Propranolol, Lidocaine and Propafenone) with low, medium and high hepatic extraction ratios (E_H) (ranging from 0.02 to 1.0) that are commercially available (Sigma-Aldrich) were selected to validate the automated method. Parallel experiments were conducted in a manual fashion to allow for comparisons between the two techniques. Additionally, 10 Pfizer model compounds with varying E_H (Table 1) in combination with the nine literature compounds were used to establish linear correlation between predicted/measured E_Hs (from automated procedure) and known in vivo E_Hs.

2. Experimental

2.1. Chemicals

Chemicals including Tolbutamide, Triazolam, Zolpidem, Nortriptyline, Chlorpromazine, Metoprolol, Propranolol, Lidocaine, and Propafenone were purchased from Sigma (Sigma-Aldrich Co., St. Louis, MO). Leibovitz's L-15 medium was obtained from Invitrogen Corp. (Grand Island, NY).

2.2. Instrument and materials

For all studies, a Tecan Genesis 200 Workstation™ (Research Triangle Park, NC) was employed. This workstation consisted of an eight-channel liquid handling arm (using standard fixed, non-disposable tips), and was equipped with a six-position 96-well aluminum orbital shaker. Temperatures in the aluminum shaker were maintained at 37 ± 0.1 °C using a waterbath. The LC–MS/MS system consisted of a Sciex API-3000 mass spectrometer from Applied Biosystems (Foster City, CA), two Series 200 Micro Pumps from PerkinElmer (Wellesley, MA), a CTC Analytics model HTS-PAL autosampler from LEAP Technologies (Carrboro, NC), and a Valco Cheminert 2 position, six port switching valve from VICI (Houston, TX). The YMC Basic HPLC columns were from Waters (Milford, MA) and the Lightning Genesis HPLC columns were from Argonaut (Foster City, CA). Ninety-six-well plates (VWR #20900-900, 0.65 mL) were obtained from VWR International (Bristol, CT).

2.3. Human hepatocytes preparation

Cryopreserved human hepatocytes were purchased from XenoTech, LLC (Lenexa, KS). XenoTech protocol and XenoTech Hepatocyte Isolation Kit (XenoTech LLC, Lenexa, KS) were used for thawing cryopreserved hepatocytes. First, 50 mL of tube A (media containing Percoll™ solution used in initial cell isolation) and 50 mL of tube B (media used to wash the isolated hepatocytes) were pre-warmed at 37 °C. Subsequently, cryopreserved human hepatocytes, at least three vials from different lots, were removed from the liquid nitrogen freezer and immediately placed in 37 °C water bath for 1.5 ± 0.25 min (for 1.5 mL vials) or 2 ± 0.25 min (for 4.5 mL vials). Vials of thawed hepatocytes were gently poured into tube A. The hepatocyte cells were gently re-suspended and centrifuged at room temperature (RT) for 5 min at 90 × *g*. The supernatant was discarded and the media in tube B was added to the cells. The resulting mixture was re-suspended and centrifuged at RT for 3 min at 60 × *g*. The supernatant was discarded and the cells were re-suspended in an appropriate volume of pre-warmed Leibovitz L-15 media. Trypan blue solution in tube C (from the hepatocyte isolation kit) was used for cell count and viability calculation. Cell viability of >80% was set as criteria in selection of hepatocytes to ensure quality of this assay.

2.4. Automated human hepatocyte assay

Custom scripts were written for Tecan Genesis 200 Workstation™ to perform the assay procedure as follows.

Incubation studies were carried out in 96-well plates using a hepatocyte pool from several individuals at a cell concentration of 0.5 million cells/mL in a total volume of 50 μL. Plates on the TECAN deck were incubated and shaken (using an orbital shaker) at 37 °C for 4 h. Cells were preincubated in plates at half of the total well volume for 30 min before addition of an equal volume of Leibovitz's L-15 medium containing 2 μM of the substrate compounds. Incubations were terminated at 0, 30, 60, 90, 120, 180 and 240 min by addition of 150 μL of ice-cold acetonitrile. Additionally, 150 μL ice-cold acetonitrile was added to control wells containing substrate, in the absence of cells, in 50 μL Leibovitz's L-15 medium at 0 and 240 min. Sample plates were vortex mixed and centrifuged at 4000 rpm for 10 min. The samples were subsequently analyzed for substrate concentration according to the LC/MS/MS conditions detailed below.

Studies were conducted on two separate days, in triplicates, to evaluate the reproducibility of this assay. Accuracy and precision of dispensed liquid volumes by the TECAN were evaluated by using the water-soluble 4-nitrophenol dye with known absorptivity at pH 7.4 at 405 nm [10] or by gravimetric analysis. The dispensing speed (100 μL/s) for hepatocytes with the fixed tips was optimized for accuracy while maintaining a minimal aspirating speed and avoiding droplets. With the dye method, absorbance in 96-well plates was read using a 96-well plate reader (EL-808, Biomek) and dispensed volumes were calculated using Lambert–Beer's law. With the gravimetric method, a 96-well plate was preweighed on a Satorius R 200D balance. In lieu of using a hepatocyte suspension, HPLC grade water was used as a surrogate for verification of targeted aspiration and dispensing volumes. The targeted volume of water (25 μL) was added to all 96 wells and the plate was reweighed and dispensed water volumes were calculated using a density of water of 0.998 g/cm^3.

2.5. Manual human hepatocyte assay

The assay procedure was carried out in an identical manner with the exception that sample aliquots were taken manually using an eight-channel pipette (Rainin-pipetman). All the pipets were calibrated regularly to ensure accuracy (within 99% of the liquid volume) and quality.

2.6. Bioanalysis/LC/MS/MS conditions

Prior to analysis, 100 μL aliquots of samples were spiked with 10 μL of internal standard (0.5 μM of a proprietary Pfizer compound that ionizes in both positive and negative mode in an ethanolic solution), vortex mixed and then centrifuged at 4000 rpm at 10 °C in an Eppendorf 5810R centrifuge. Aliquots (5 μL, flushed loop injection) of sample were analyzed using a step gradient HPLC method utilizing mobile phase A (water–acetonitrile–formic acid (95:5:0.001, v/v/v)) and mobile phase B (water–acetonitrile–formic acid (10:90:0.001, v/v/v)), which were mixed in a static tee mixer before the autosampler (the stock concentration of formic acid was 88%). Chlorpromazine, Naloxone, Nortriptyline, Propafenone, Propranolol, Tolbutamide, Triazolam, Verapamil, Zolpidem, PF9 and PF10 samples were analyzed on a Waters YMC Basic S-5 guard cartridge (2.0 mm × 20 mm, 5 μm particle size). Lidocaine, Metoprolol, Pfizer compound 1 (PF1), PF2, PF3, PF4, PF5, PF6, PF7, and PF8 samples were analyzed on a Lightning Genesis C18 column (2.1 mm × 50 mm, 3 μm particle size). The column was plumbed in line between the autosampler and the switching valve which directed flow to either waste or to the mass spectrometer. Initially, the samples were loaded onto the column with 100% mobile phase A at 350 μL/min with the flow diverted to waste. After a 0.8 min wash step with mobile phase A, the valve was switched and the pumps stepped to 100% mobile phase B at 400 μL/min such that the column was eluted with mobile phase B into the mass spectrometer. At a particular time (2.0 min total run time for YMC Basic and 2.5 min total run time for Lightning Genesis), the valve was switched back to its initial position and the pumps stepped back to 100% mobile phase A at 350 μL/min to allow for re-equilibration of the column. The total run times were 2.5 min for the YMC Basic and 3.5 min for the Lightning Genesis. The mass spectrometer was controlled with MassChrom 1.1 software and equipped with a Turbo Ionspray source. Peak area ratios were calculated by dividing the analyte peak area by the internal standard peak area using TurboQuan 1.0 software. Optimal *m/z* and collision energies were determined through flow injection analysis. Collision voltages ranged from ±35 to ±45 V. The following multi-reaction monitoring (MRM) transitions were monitored for the analytes of interest:

Chlorpromazine: (+) 318.8 → 86.1; Lidocaine: (+) 234.8 → 86.1; Metoprolol: (+) 267.8 → 116.2; Naloxone: (+) 328.4 → 253.4; Nortriptyline: (+) 264.0 → 117.2; Prednisone: (+) 359.2 → 267.2; Proprafenone: (+) 341.7 → 116.2; Propranolol: (+) 261.1 → 116.2; Tolbutamide: (−) 269.2 → 170.1; Triazolam: (+) 343.1 → 308.1; Verapamil: (+) 455.4 → 165.2; Zolpidem: (+) 307.8 → 235.2; PF1: (+) 413.0 → 194.1; PF2: (+) 330.0 → 122.1; PF3: (+) 296.9 → 160.2; PF4: (+) 328.1 → 160.2; PF5: (+) 451.8 → 344.1; PF6: (+) 409.1 → 238.1; PF7: (+) 307.4 → 220.1; PF8: (+) 254.2 → 197.9; PF9: (+) 442.1 → 198.0; PF10: (+) 475.2 → 99.7.

2.7. Evaporation test

Experiments were performed to investigate the possibility of sample evaporation during the 4 h incubation on the Tecan deck. At 37 °C, 50 μL of Leibovitz's L-15 medium was added to each well in a 96-well plate and subsequently 150 uL of acetonitrile was added at various time points (0, 30, 60, 90, 120, 180, and 240 min). Following the 240 min time point, 10 μL of internal standard was added to each well and the

plate was sealed and analyzed by LC/MS/MS for internal standard response. Each time point was carried out in 12 replicates.

2.8. Assay quality control

Within each TECAN human hepatocyte experiment, six 96-well plates were processed, 48 compounds were run and approximately 600 samples were generated. Along with the discovery compounds, three positive controls (Propranolol, Triazolam (was recently changed to Verapamil), and Naloxone) are used to allow for a retrospective analysis of the robustness of this assay.

2.9. Data analysis

To determine the hepatic intrinsic clearance (CL_{int}), the half-life ($t_{1/2}$) was calculated from a log linear plot of peak area ratio versus time using WinNonLin™ (non-compartmental, model 201, version 3.0, Pharsight Corporation, Mountain View, CA) and the following equations were employed [9]:

$CL_{int} = (0.693/t_{1/2}) \times$ (g liver/kg body) $\times$ (mL incubation/cells incubation) $\times$ (cells/g liver)

$CL_{blood} = CL_b = Q(1 - e^{(-CL_{int}/Q)})$ (parallel-tube model without including fraction unbound).

$$E_H = CL_b/Q$$

where CL_b, blood clearance; Q, liver blood flow. For humans, the following values apply: $Q = 20$ mL/min/kg; 21 g liver/kg body; 1.2×10^8 cells/g liver.

The success criteria for this assay are that the predicted E_H should be within two-fold of the known in vivo human E_H for the 19 compounds tested [11].

3. Results and discussions

3.1. Cell viability with manual and automated assays

The viability of hepatocytes after pippetting with the TECAN® liquid handler was similar to that after manual pipetting (85%, Fig. 1), while maintaining pipetting accuracy and precision. Volume pipetting accuracy for a targeted hepatocyte dispensing volume of 25 μL was 24.57 ± 0.08 μL and the relative error was less than 5% as determined by the gravimetric method (Table 2). Similarly, using the dye method, the dispensed volume was 25.8 ± 1 μL and the relative error was also less than 5% (Table 3). These results demonstrate that human hepatocytes maintained viability up to 4 h with adequate pipetting accuracy and precision. While the literature references on the viability of hepatocytes as a function of time varies, four hours were deemed to be appropriate for the conduct of high throughput hepatocyte screen in our drug discovery.

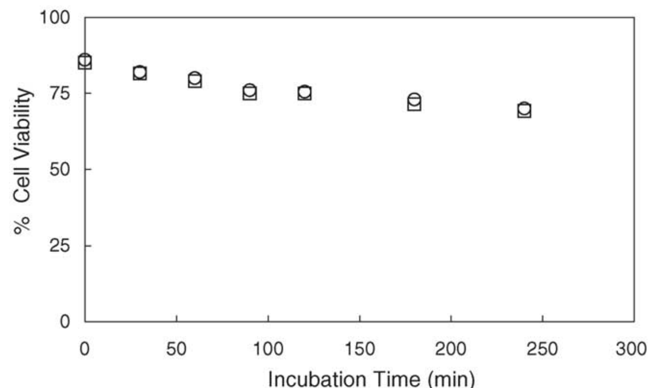

Fig. 1. Viability of cryopreserved hepatocytes pipetted by (□) automated and (○) manual methods.

Table 2
Gravimetric volume determinations for the accuracy of the robotic pipetting (25 μL of water was added to 9 columns, 8 rows, and 72 wells)

Run#	Day 1 (μL H_2O)	Day 2 (μL H_2O)	Day 3 (μL H_2O)
R1	25.10	23.78	24.26
R2	24.15	24.65	24.89
R3	24.11	24.69	24.78
R4	24.08	23.95	24.46
R5	23.66	25.59	24.91
R6	25.51	24.34	24.66
Mean ($n = 6$)	24.494	24.551	24.660
Standard deviation	0.666	0.606	0.257
Volume (μL H_2O added)	25	25	25
Relative error (%)	2.02	1.79	1.36
CV (%)	2.72	2.47	1.04
Mean ($n = 18$)		24.57	
Standard deviation		0.08	

Table 3
Determinations of dispense volume by Tecan Genesis™ liquid handler in individual wells using 4-nitrophenol absorbance ($A = \varepsilon cd$, via 96-well reader)

	μL added								
	1	2	3	4	5	6	7	8	9
A	49.6	48.6	48.8	24.7	25.1	25.9	6.2	6.7	6.3
B	50.4	50.3	50.0	27.2	27.7	27.5	6.5	6.4	6.8
C	48.1	48.0	47.8	26.5	26.3	26.2	5.9	6.0	5.7
D	47.1	47.8	46.9	25.2	25.3	25.8	6.5	7.2	5.5
E	47.0	48.0	48.9	24.8	24.4	24.2	6.9	7.1	6.0
F	49.0	47.8	49.9	26.7	25.9	26.0	6.5	5.7	5.6
G	48.7	48.6	48.1	26.1	25.2	25.9	6.2	6.2	6.8
H	49.0	48.7	48.8	24.4	26.0	25.5	5.6	5.8	5.0

Average (μL) ($n = 24$)	48.6	25.8	6.2
Standard deviation	1.0	1.0	0.55
Target (μL)	50	25	6
Relative error (%)	2.8	3.2	3.3
CV (%)	2.03	3.71	8.89

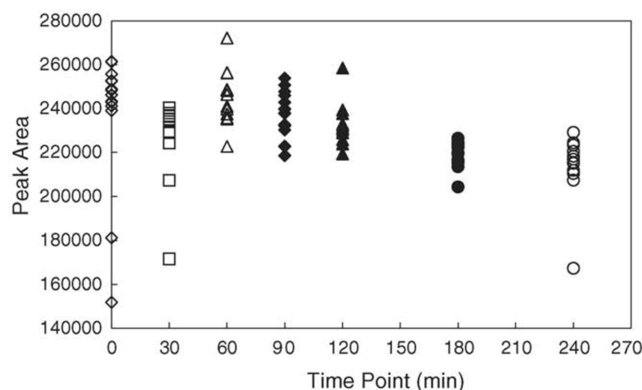

Fig. 2. Evaporation test of internal standard during the 240 min incubation on Tecan at 37 °C: (◇) 0 min time point; (□) 30 min time point; (△) 60 min time point; (♦) 90 min time point; (▲) 120 min time point; (●) 180 min time point; (○) 240 min time point.

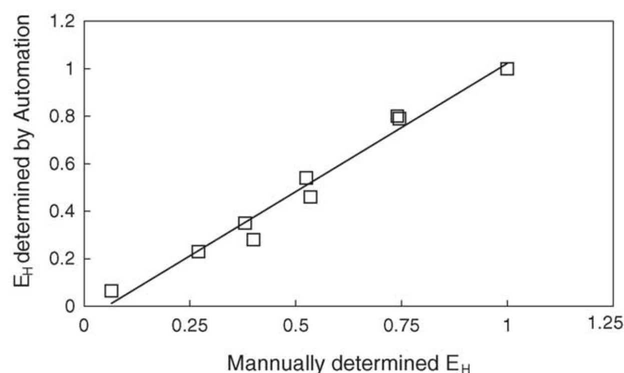

Fig. 3. Correlation between E_H of model compounds obtained by automated and manual methods ($R^2 = 0.97$). All data are mean ± S.D. with $n = 3$ or 6.

3.2. Evaporation test

Since the hepatocyte assay is performed under an open environment at 37 °C, significant evaporation can cause variation of analyte concentration during the 4 h incubation. In order to investigate this possibility and its potential impact upon experimental results, experiments were designed to test the effects of 4 h plate storage on the internal standard response. The experiments were designed in such a way as to mimic the conditions on the TECAN during the course of an actual hepatocyte run (see Section 2 for details). Based on the final results displayed in Fig. 2, there is a small trend toward increasing peak area with increasing time of evaporation (for example, 0 min time points with potential evaporation time of 240 min had relative higher peak areas than those of 240 min time points with no evaporation time), but the difference between time points was all less than ∼10%. Based on the data, it is concluded that the impact of evaporation on the TECAN is minimum and if present, will be within the acceptable limits (±15%) for the purpose of this high throughput assay.

3.3. Validation with commercial compounds

Nine commercial compounds with a range of literature E_H values were chosen to validate the high throughput human hepatocyte screen (Table 1). Two experiments (experiments 1 and 2) (see Tables 4 and 5) were performed on different days with each experiment being carried out in triplicates within a day. In general, concentration–time plots of substrate disappearance were linear with values of $r^2 \geq 0.85$ (data not shown).

Results from the manual experiments and automated TECAN Genesis™ experiments (in 96-well plates) for these nine compounds are displayed in Tables 4 and 5, respectively. The data (Tables 4 and 5) indicates that the measured E_Hs are comparable to the literature values (i.e., within two-fold difference except for Tolbutamide whose half-life is in the range of 2000 min) for both manual and automated procedures. In addition, plot for manually measured E_H versus automation predicted E_H is highly correlated ($r^2 = 0.97$, Fig. 3). Furthermore, the inter- and intra-day variability for both assays (manual and automated) were acceptable for decision-making in early discovery in support of lead optimization.

3.4. Validation with in-house compounds

Ten in-house compounds (Pfizer compounds) were chosen to further validate the HT human hepatocyte assay (Table 1). The correlation between predicted (using measured hepatocyte results) human mean E_H and known in vivo human E_H (as shown in Table 6) indicated that 8 of the 10 compounds

Table 4
Extraction ratios obtained via manual pipeting method for human hepatocyte screen

Compound	E_H (experiment 1)	E_H (experiment 2)	Mean E_H (±S.D.)	CV (%)
Tolbutamide	0.06 (±0.02)[a]	0.07 (±0.005)[a]	0.065 (±0.01)[b]	0.02
Triazolam	0.27 (±0.10)	N/D	0.27	N/A
Zolpidem	0.38 (±0.15)	N/D	0.38	N/A
Nortriptyline	0.43 (±0.05)	0.37 (±0.05)	0.40 (±0.04)	10
Chlorpromozine	0.57 (±0.067)	0.50 (±0.03)	0.535 (±0.05)	9
Metoprolol	0.53 (±0.16)	0.52 (±0.27)	0.525 (±0.007)	1
Propranolol	0.80 (±0.08)	0.69 (±0.056)	0.745 (±0.08)	11
Lidocaine	0.74 (±0.11)	N/D	0.74	15
Propafenone	1.00 (±0.21)	1.00 (±0.11)	1.00 (±0.00)	0

N/D, not determined; N/A, not applicable.
[a] Mean ± S.D., $n = 3$.
[b] Mean ± S.D., $n = 6$ (three measurements from experiment 1 and three measurements from experiment 2).

Table 5
Extraction ratios obtained via the automated human hepatocyte screen

Compound	E_H (experiment 1)	E_H (experiment 2)	Mean E_H (±S.D.)	CV (%)
Tolbutamide	0.07 (±0.02)[a]	0.06 (±0.00)[a]	0.065 (±0.01)[b]	15
Triazolam	0.16 (±0.04)	0.29 (±0.09)	0.23 (±0.09)	39
Zolpidem	0.26 (±0.006)	0.43 (±0.04)	0.35 (±0.12)	34
Nortriptyline	0.25 (±0.09)	0.31 (±0.09)	0.28 (±0.04)	14
Chlorpromozine	0.44 (±0.03)	0.47 (±0.07)	0.46 (±0.02)	4
Metoprolol	0.59 (±0.10)	0.50 (±0.05)	0.54 (±0.06)	11
Propranolol	0.82 (±0.04)	0.76 (±0.01)	0.79 (±0.04)	5
Lidocaine	0.71 (±0.08)	0.88 (±0.01)	0.80 (±0.12)	15
Propafenone	1.00 (±0.00)	1.00 (±0.00)	1.00 (±0.00)	0

[a] Mean ± S.D., $n = 3$.
[b] Mean ± S.D., $n = 6$ (three measurements from experiment 1 and three measurements from experiment 2).

Table 6
In-house compounds (10 total): predicted human E_H (obtained via the automated human hepatocyte screen) vs. in vivo human E_H

In-house compound	E_H (experiment 1)	E_H (experiment 2)	Predicted human E_H	CV (%)	In vivo mean (E_H)
PF1	0.88 (±0.03)[a]	0.90 (± 0.04)[a]	0.89 (±0.01)[b]	1	0.52
PF2	0.77 (±0.08)	0.61 (± 0.02)	0.69 (±0.12)	17	1.00
PF3	0.97 (±0.00)	0.88 (± 0.04)	0.93 (±0.06)	6	0.73
PF4	0.54 (±0.02)	0.36 (± 0.04)	0.45 (±0.13)	29	0.80
PF5	0.32 (±0.02)	0.19 (± 0.03)	0.26 (±0.09)	35	0.06
PF6	0.05 (±0.02)	0.09 (± 0.01)	0.07 (±0.03)	43	0.35
PF7	0.04 (±0.00)	0.03 (± 0.00)	0.04 (±0.01)	25	0.02
PF8	0.24 (±0.04)	N/A	0.24		0.16
PF9	0.24 (±0.03)	N/A	0.24		0.22
PF10	0.67 (±0.02)	0.56 (±0.07)	0.62 (±0.08)	13	0.49

N/A, not available.
[a] Mean ± S.D., $n = 3$.
[b] Mean ± S.D., $n = 6$ (three measurements from experiment 1 and three measurements from experiment 2).

met our predetermined success criteria (which is that the measured E_H should be within two-fold of the known in vivo E_H). In addition, the inter- and intra-day reproducibility of this assay was acceptable (shown in Table 6) for decision-making in early discovery. Currently, it is not clear why PF5 and PF6 failed in this assay and investigation is underway to explore potential causes of this failure. One of the hypotheses is that extra-hepatic tissues may play a role in the overall clearance of PF5 and PF6. Current effort is to search for Pfizer internal research reports on PF5 and PF6 to further understand their clearance mechanisms.

3.5. Assay application in early drug discovery

A high throughput human hepatocyte assay has been successfully implemented as a part of pharmacokinetic characterization of NCEs in drug discovery programs. To date, hepatic intrinsic clearance data has been generated for approximately 1200 discovery compounds. Within each production run, three positive controls are used along with NCEs to allow for a retrospective analysis of the robustness of this assay. The three controls are Propranolol (2D6 substrate), Triazolam (recently changed to Verapamil due to the fact that Triazolam is a controlled substance) (3A4 substrate), and Naloxone (UGT substrate). If the measured E_Hs (for the positive controls) are in agreement with their literature values (% difference ≤ 30), the screening run is considered to be within specifications. In 2002, there were approximately 10 production human hepatocyte runs performed. Within each of the production run, E_H data were generated for the three control compounds (displayed in Table 7). In the case of Verapamil, information was only available for last seven runs, due to the fact that Triazolam was used as one of the controls for the first three runs. Overall, good reproducibility was observed for the three controls with CV less than 20%. Additionally,

Table 7
E_H values generated for three controls (Propranolol, Verapamil, and Naloxone) from 2002 in-house production runs

Number of production runs	E_H of Propranolol	E_H of Verapamil	E_H of Naloxone
1	0.56	N/A	1.00
2	0.71	N/A	0.98
3	0.87	N/A	0.97
4	0.67	0.92	0.99
5	0.90	0.98	0.98
6	0.75	0.93	0.94
7	0.92	0.95	0.98
8	0.86	0.85	0.96
9	0.82	0.90	0.95
10	0.60	0.95	0.94
Average of E_H	0.77	0.93	0.97
Standard deviation	0.13	0.04	0.02
CV (%)	16	4	2
In vivo human E_H	0.70	0.95	0.86

N/A, not available.

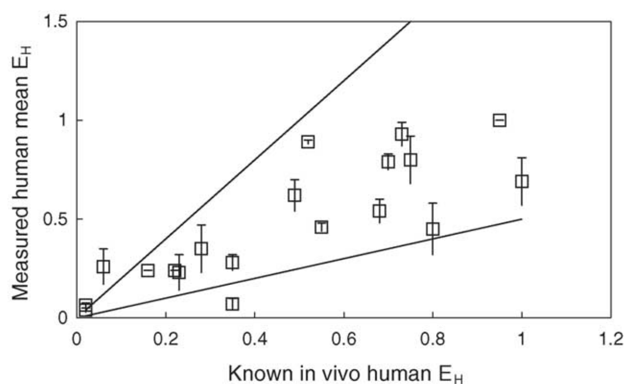

Fig. 4. Extraction ratios for model and Pfizer compounds. E_H for 17 out of 19 selected compounds was within two-fold of known in vivo E_H [15–23]. All data are mean ± S.D. with $n = 3$ or 6. The two lines represent the two-fold range of the known in vivo human E_H values.

the average E_H values for the three controls correlates very well with the corresponding in vivo human E_H values (% difference between measured E_Hs and in vivo human values was <13).

4. Conclusions

A TECAN Genesis 200 Workstation™ equipped with: (1) a liquid handling arm with eight standard fixed tips; (2) a temperature controlled orbital shaker, in which incubation conditions can be precisely controlled; and (3) TECAN's Gemini® liquid handling software, was used to perform human hepatocyte assay to enable determination of human metabolic intrinsic clearance. Pipetting steps for cryopreserved hepatocytes were optimized to minimize the loss in cell viability by setting aspirating and dispensing speeds with TECAN's Gemini® software. The accuracy of hepatocyte liquid dispensing volume was verified using 4-nitrophenol dye and compared to manual pipetting. Hepatocyte consumption was minimized to reduce assay costs and the assay was programmed to allow flexibility in screening between 8 and 48 compounds/run with up to 10 time points and two chemical stability controls. As indicated in Fig. 4, 17 of 19 tested compounds (both commercial and in-house) have met our success criteria. The predictive power of this assay is 90% based on this set of 19 compounds.

4.1. Limitations of the assay

As indicated in the equation (to calculate hepatic clearance), this assay does not take into consideration of the effect of protein binding. Overall, the predicted human values (from this assay) correlates reasonably well with the known/literature human values without including a correction for protein binding data. Additionally, the assay will only accurately predict in vivo clearance via hepatic metabolism. It is well known that the liver is the major site in the body for xenobiotic metabolism [12]. However, tissues, such as intestinal and kidney, can also actively metabolize many xenobiotics [13]. In addition, clearance can be affected by transporter-mediated influx and efflux [12,14]. Therefore, if the clearance of a compound in human is not metabolism related (such as transporter-mediated influx and efflux) or is metabolized via non-hepatic routes, then the in vitro assay is expected to underestimate (or overestimate in the case of transporter-mediated influx) in vivo clearance.

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