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Comparative T4-glucuronidation Assay in Primary Human and Rat Hepatocyte in the TruVivo System for Generation of a Historical Control Database

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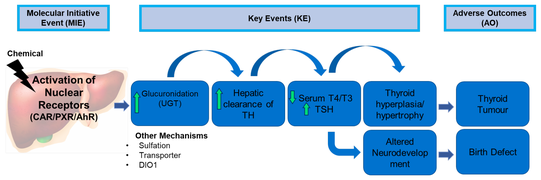
INTRODUCTION:

- Increased hepatic thyroid hormone (TH) metabolism due to induction of glucuronidation via Uridine 5'-diphospho-glucuronosyltransferase (UGTs) is the main key event in the TH perturbation mode of action (MoA)[1,2,3].
- Previously, Corteva collaborated with LifeNet Health to optimize a new approach methodology (NAM) utilizing TruVivo, an *invitro* hepatic system, to compare liver-mediated TH perturbation in response to known AhR/CAR/PXR agonists[4].
- Currently there are no guidelines and no acceptance criteria for an assay that evaluates the *in vitro* comparative quantitative analysis of liver mediated TH disruption among rats and humans.
- The current study is focused to evaluate the quantitative variance of T4G among the two species (rat and human) in a set of nine primary hepatocytes lots after exposing to previously used reference compounds. The data was statistically evaluated after three independent repeats with the objective to generate a minimal historical database that can determine the range of variation and provides an acceptance criteria for the assay.



Chemical exposure cause activation of NR activation (like CAR/PXR/AhR), which is a molecular initiated event that leads to a sequence of key events in liver including the induction of phase II metabolism, DOI1, up-regulation of transporters., increased hepatic clearance of TH, low level of TH in serum, increased TSH, thyroid histopathological changes like follicular hyperplasia, hypertrophy, and ultimately the adverse outcome of thyroid cancer. Correspondingly, maternal low level of serum TH irrespective of increased TSH leads to altered neurodevelopment and birth defect[5].

Material and Method:

Cryopreserved primary Hepatocytes from 6 individual human donors (3Male/3Female) and 3 pooled Sprague Dawley rat lots (2Male/1Female) that were cultured in TruVivo.

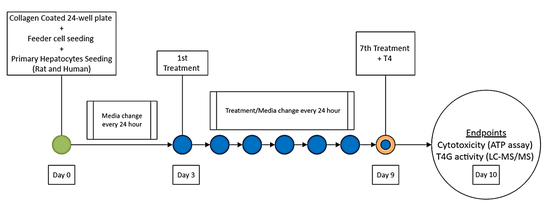
Rat Lot CoA information
Donor information

No	strain	Cat name	lot no	no of donors	age (weeks)	Sexes
1	SD	RTCP10	RS1006	8	11	M
2	SD	RTCP10	RS1007	8	9	M
3	SD	RTCP20	RS1005	8	9	F

Human Donors CoA information

no	strain	Donor name	age	BMI	Sexes	Cause of death
1	Human	1921756-02	56	29	M	CVA/stroke
2	Human	1922211-02	40	28	M	Anoxia
3	Human	1813386-01	42	31	M	Stroke
4	Human	1916422-01	48	24	F	Resp Arrest
5	Human	2113352-01	36	31.6	F	Anorexia
6	Human	2214423-01	28	20	F	Anorexia

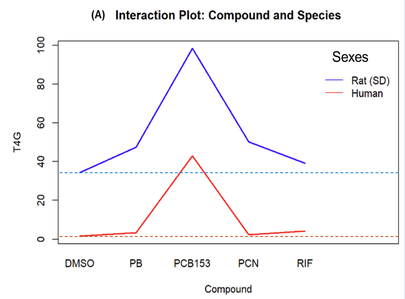
- The TruVivo cultures were repeatedly exposed daily to nuclear receptor activating reference compounds (Polychlorinated biphenyl 153 (PCB153; 30 µM),Pregnenolone 16α-carbonitrile (PCN; 20 µM), Phenobarbital (PB; 500 µM) and Rifampicin (RIF; 10 µM) at non-toxic concentrations (cytotoxicity <20%) for 7days.
- Physiologically relevant level of T4 (0.05 µM rat, 0.1 µM human) were added with the 7th exposure. T4 glucuronide (T4G) was quantified in incubation medium up to 24 hours post-T4 addition using a validated LC-MS/MS method.
- The experiment was independently repeated three times.
- The LC-MS/MS method was run on an Agilent 1290 Infinity II HPLC system coupled with an Agilent G6495C triple-quadrupole MS. Individual analyte concentrations in the samples were quantitated using calibration curves and standards (0.03-150ng/mL). T4G values (nM) were normalized to pmol/10^6cells/24hrs. Comparative compound response relative to vehicle control was calculated as ΔT4G .
- All statistical analysis was conducted to identify major variations in species, gender, lot, and run. Additionally, analysis were conducted to determine control limits based on the HCD variability among runs and lots, while taking into account the effect of outliers



Schematic Study Design of T4-glucuronidation assay

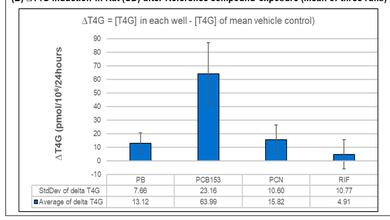
Results and Discussion

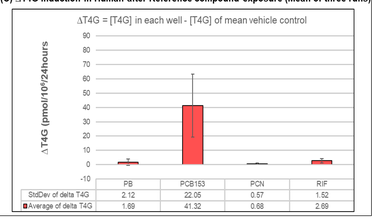
Descriptive analysis: Quantitative difference among species



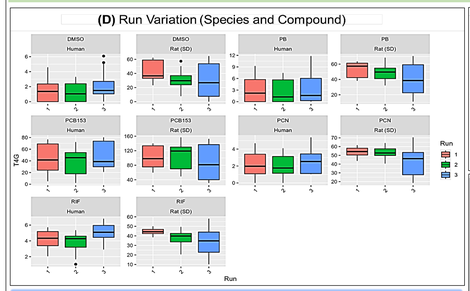
- Vehicle control T4G mean data showed that rats (34.3±18.3 pmol/10^6/24hr) have higher level (~20-fold) of T4G compared to humans (1.62±1.6 pmol/10^6/24hr). A quantitative species difference was illustrated in all exposure groups (**A**).
- PCB153 caused increased ΔT4G induction in both rat (64±23.2 pmol/10^6/24hr) and human (41.3±22 pmol/10^6/24hr) (**B & C**).
- PB and PCN caused increased ΔT4G induction of 13.1±7.7 & 15.8±10.6 pmol/10^6/24hr respectively in rat (**B & C**).
- RIF caused slight increase ΔT4G induction of 2.7±1.5 pmol/10^6/24hr in human (**B & C**).

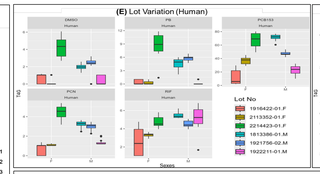
(B) ΔT4G induction in Rat (SD) after Reference compound exposure (Mean of three runs) (C) ΔT4G induction in Human after Reference compound exposure (Mean of three runs)

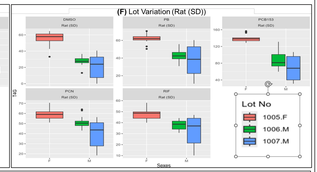


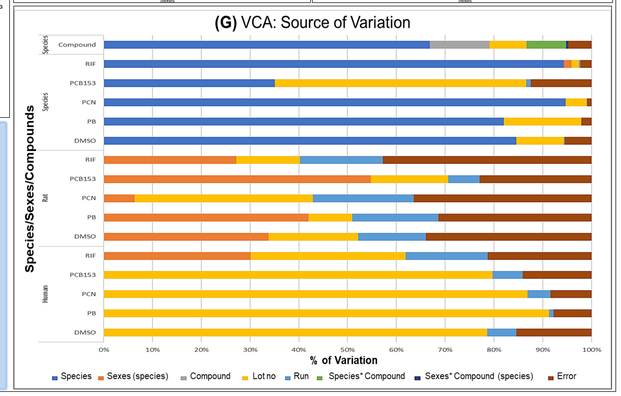


Source of Variation (run, species, sexes & compound)



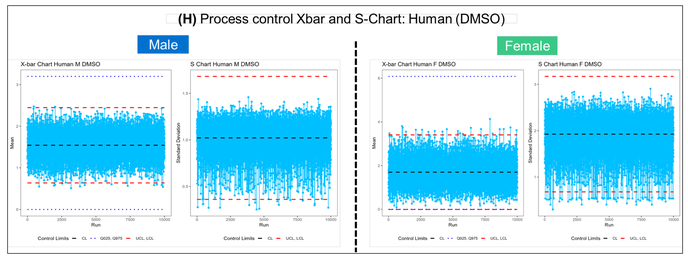


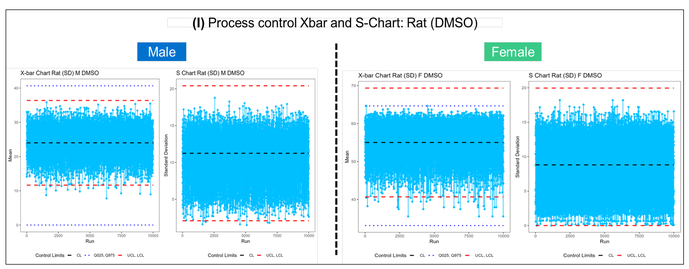


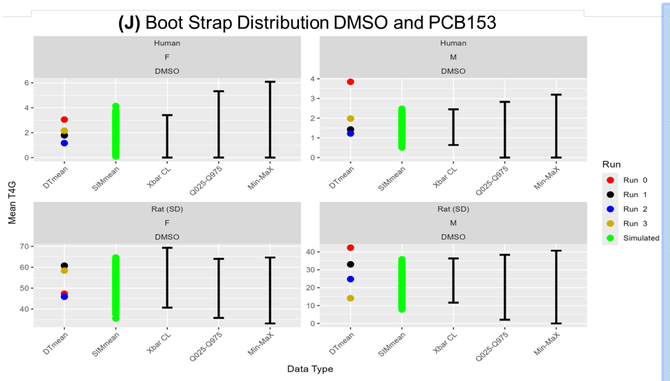


- Variability within each runs was evidently seen in both species and in all exposure groups (**D**). Variation component analysis (VCA) estimated the major source of variation in both species combined was due to species (35-92%) and lot (2-50%) (**G**).
- Variation in tested population of human was noticed among donor to donor (**E**), VCA estimated the major source of variation in human population was due to lots (32-92%)and run (2-10%) (**G**).
- Variation in tested population rat population was also evident among lot to lot (**F**), VCA estimated the major source of variation in rat population was due to lots (10-35%), sexes (5-54%) and run (8- (**G**).

Bootstrap sampling to generate Control Limits and assessment of a test run







(K) Control Limits on DMSO and PCB153 (species and sexes)

Species	gender	Compound	n	Xbar-LCL	Xbar-CL	Xbar-UCL	Q025	Q975
Human	F	DMSO	12	0.00	1.70	3.41	0.00	6.08
Human	M	DMSO	12	0.64	1.54	2.45	0.00	3.20
Human	F	PCB153	15	19.14	38.49	57.84	2.28	78.90
Human	M	PCB153	15	31.22	47.39	63.55	15.91	76.82
Rat (SD)	F	DMSO	4	40.67	55.01	69.35	33.12	64.65
Rat (SD)	M	DMSO	8	11.65	24.02	36.38	0.00	40.67
Rat (SD)	F	PCB153	5	127.55	139.58	151.61	128.61	156.76
Rat (SD)	M	PCB153	10	51.28	77.59	103.91	31.11	131.20

- Bootcamp sampling was generated by simulated run from a random sample with 20% variability replacement was taken and considered it as a new run. In total 10,000 runs were generated to create HCD and control limits(CL) in both species and sexes separately (**H & I**).
- The CL methods (Xbar, simulated mean (SIMean), Quantile 25-97.5 (Q025-Q975) and Min-Max) were calculated for limit observation in all exposure groups (**J &K**). Xbar CL is symmetric and influenced by skewed distribution. While Quantile CL is asymmetric and based on data distribution.
- Test run "0" data was compared with the generated CL (**red dot (J)**. It was noticed that the run 0 fall outside of CL in some instances (e.g., Human and rat male DMSO), suggested that the CL are more conservatively estimated and only become more stable with additional repeated run.
- **Recommendation:**
  - Updated HCD after every conducted study.
  - HCD will be reviewed every year to recommend on a CL criteria.
  - Future study will be conducted on the same lot/donors that are tested in this study.
  - The established HCD was established on the study design used in this study. Any change in method (e.g., addition of new lot/donor) in future will require to demonstrate no impact in assay performance by illustrating CL to be within the range of established HCD.

Conclusion :

- Cell viability in all groups remained >80%, suggesting that the T4G induction by the reference control was without compromising the health of the hepatocytes.
- High T4G in rat compared to the human levels, suggested that there is Quantitative species difference among the two.
- PCB153 showed high induction of T4G in both species, suggesting PCB153 as a definite positive control for the assay.
- PCN response in Rat only and RIF response in human only suggested species relevant response.
- Variability among each species population was majorly due to lots, suggested an inherit variability of the assay.
- CL are likely to be stable as additional runs will be included to the HCD.
- Incorporating a HCD in the assay design has established confidence regarding assay repeatability. HCD review and maintenance for future studies will help to demonstrate reproducibility and acceptance criteria.

References & Acknowledgments:

Walter et al., 2025; 2. Richardson et., al 2014; 3. Baze et al., 2024; 4. Raza et al., SOT2024; 5. Noyes et al., 2019

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