# High Throughput Determinations of Critical Dosing Parameters

3437/P127
Society of Toxicology Annual Meeting
New Orleans, LA
March 13-17, 2016

Chantel I. Nicolas[1], Brandall L. Ingle[2], Maria Bacolod[3], Jon Gilbert[3], Barbara A. Wetmore[4], Caroline L. Ring[5,6], R. Woodrow Setzer[5], Rogelio Tornero-Velez[2], Matthew T. Martin[5], and John F. Wambaugh[5*]

1. Biomedical/Biotechnology Research Institute, North Carolina Central University, Durham, NC, United States
2. National Exposure Research Laboratory, U.S. EPA, Research Triangle Park, NC, United States
3. Cyprotex US LLC, Watertown, MA, United States

1. ScitoVation, LLC, Research Triangle Park, NC, United States
2. National Center for Computational Toxicology, U.S. EPA, Research Triangle Park, NC, United States
3. Oak Ridge Institute for Scientific Enrichment, Oak Ridge, TN, United States.

www.epa.gov/ncct/expocast

Chantel I. Nicolas | cnicolas@nccu.edu | 919-530-7015

www.epa.gov

## Abstract

High throughput toxicokinetics (HTTK) is an approach that allows for rapid estimations of TK for hundreds of environmental chemicals. HTTK-based reverse dosimetry (i.e, reverse toxicokinetics or RTK) is used in order to convert high throughput in vitro toxicity screening (HTS) data into predicted human equivalent doses, which can be linked with biologically relevant exposure scenarios. Therefore, HTTK provides critical data in order to prioritize the risk for thousands of chemicals that lack TK data. The unbound fraction of a chemical in plasma ($F_{ub}$) is a critical HTTK parameter that can be measured in vitro. However, for current methods whereby $F_{ub}$ is measured at 100% plasma concentration, $F_{ub}$ is below the limits of quantitation (LOQ) for high throughput analytical chemistry for chemicals that bind strongly to plasma, and therefore cannot be quantified. In order to quantify Fub, a novel method was implemented for 85 strategically selected chemicals: $F_{ub}$ was measured at 10%, 30%, and 100% of physiological plasma concentrations using rapid equilibrium dialysis assays. Chemicals were selected based on their capacity to be potent in vitro estrogen signaling disruptors (Rotroff *et* al. 2014), having NHANES data, or either having no HTTK data or a failed $F_{ub}$ assay. Including plasma concentrations substantially lower than physiological levels allows the direct measurement of unbound chemical concentrations. The consequent Fub estimates at lower protein concentration can be extrapolated to physiological levels. At 100% plasma concentration, assays yielded values below LOQ for 34 chemicals. $F_{ub}$ could be quantified for 12 of these 34 chemicals at 10% and/or 30% plasma concentrations, which suggests that assay failure at 100% plasma concentration was caused by plasma protein binding for these chemicals. For the remaining 22 chemicals, assay failure may be due to chemical insolubility, susceptibility to enzymatic or other degradation, and ability to bind to RED device constituents such as assay plate walls or dialysis membrane. As a result of using this new approach, ~35% of missing $F_{ub}$ values were captured and would have been missing with the use of previous HTTK protocols. *This abstract does not necessarily reflect U.S. EPA policy.*

## Introduction

**Introduction:**

• One major challenge facing the use of high-throughput screening (HTS) *in vitro* assay methods is relating *in vitro* bioactive doses to their equivalent human *in vivo* doses in a rapid and scaled manner.[1-4]

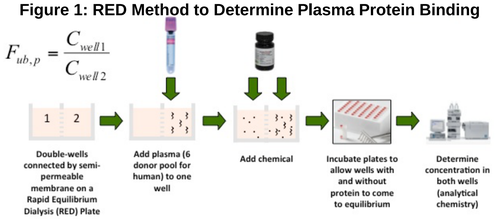
• A critical parameter for many *In vitro* to *in vivo* extrapolation (IVIVE) methods is fraction of unbound chemical in plasma ($F_{ub}$). [5-6]

• Rapid equilibrium dialysis[7] (RED) assays depicted in Figure 1, have been used to quantify $F_{ub}$ at 100% physiological plasma concentrations.

• However, $F_{ub}$ values are often not determined by the RED assay due to unbound chemical concentrations being below the limit of quantitation (LOQ).

• This project analyzes a new method for determining $F_{ub}$ at various reduced plasma concentrations.

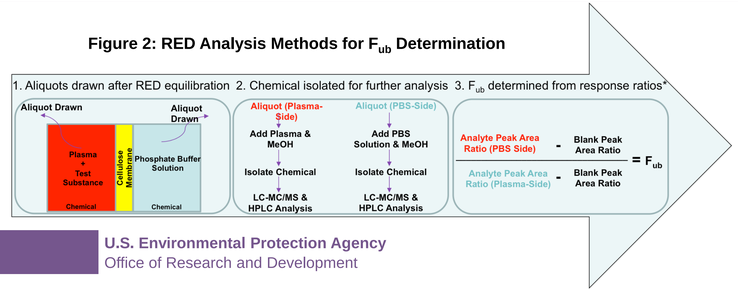
**Figure 1: RED Method to Determine Plasma Protein Binding**




**OBJECTIVE**:
**Increase the total number of chemicals for which IVIVE can performed by: determining $F_{ub}$ for chemicals whose values are below LOQ by varying the plasma concentration used in rapid equilibrium dialysis (RED) assays.**

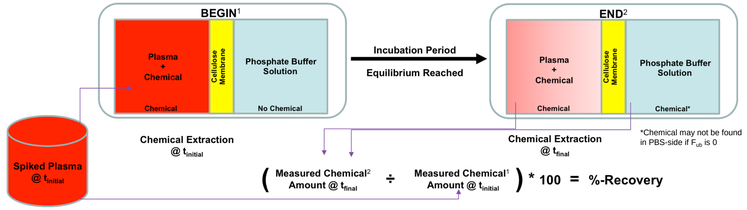
## Methods I: Determine Fraction Unbound ($F_{ub}$)

**Figure 2: RED Analysis Methods for $F_{ub}$ Determination**




## Methods II: Chemical Recovery (%-Recovery)

**Figure 3: RED Analysis Methods for Determining %-Recovery**




**Methods I:**

• Figure 2 illustrates a more detailed experimental procedure (Step 1 and 2) and summarizes how $F_{ub}$ is determined analytically. In the experiment, three plasma concentrations were used: 10%, 30%, and 100% for 85 commercial chemicals.

• For each chemical, peak area ratios were measured in duplicates for four different sample types: blank samples, plasma samples at t = 0, plasma samples at t = final (equilibrium reached), and phosphate buffer solution samples at t = final. These 8 measurements were taken at three different plasma concentrations, for a total of 24 measurements for each chemical.

**Methods II:**

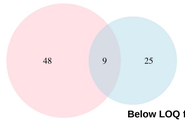
• Figure 3 describes a method determining efficacy. After plasma is spiked with a chemical, some of it is placed in the plasma side of the RED device (left well), while some of it goes through a chemical extraction process.

• At the end of the experiment, solutions from both sides of the RED device go through a chemical extraction process. The final amount is divided by the initial amount to yield the amount of chemical that is recovered from the experiment (%-Recovery).

## Results & Discussion

**Figure 4: Chemicals Below LOQ at 100% [Plasma]**

**A. Above LOQ for 30% [Plasma]**




**Below LOQ for 100% [Plasma]**

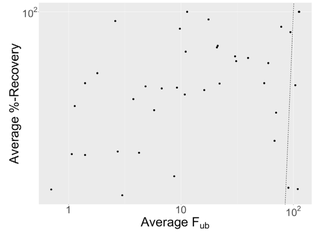
**B. Above LOQ for 10% [Plasma]**


**Below LOQ for 100% [Plasma]**

•Figures 4A and 4B show the overlap of chemicals for which $F_{ub}$ was not detectable at 100% plasma concentration ([plasma]) but were detected at 30% and 10% [plasma]. As a result of this experiment, 22 additional $F_{ub}$ values were captured (14 unique chemicals).

•In this analysis, a chemical below LOQ is defined as one whose Fub has either not be quantitated or has been quantitated as being equal to zero.

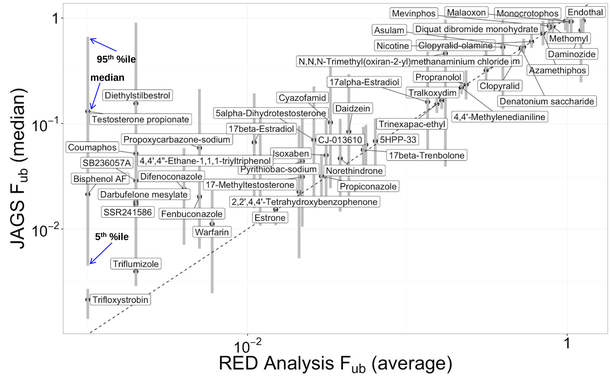
**Figure 5: $F_{ub}$ Quantitation and %-Recovery**




There are 48 unique chemicals for which $F_{ub}$ and %-recovery were determined at each plasma concentration. Figure 5 illustrates the lack of correlation between $F_{ub}$ values and %-recovery. For this set of chemicals, the correlation between the two parameters is 17% overall across the three plasma concentrations. While there is no meaningful relationship between $F_{ub}$ measurements and %-recovery, there exists high recovery percentages (>85%) for a range of unbound fractions. In general, low percent recovery suggests that $F_{ub}$ measurements for certain chemicals are potentially underestimated or overestimated due to various types of degradation or assay plate wall binding interactions.

## Bayesian Hierarchical Model for Inferring $F_{ub}$

**Figure 6: JAGS Model for Inferring $F_{ub}$ from RED Assays**




• The ability to infer $F_{ub}$ on a large scale is crucial for high throughput assessments. However, it is important to identify sources of systematic error and then use optimal methods to quantify uncertainty. A Bayesian model was employed (using 'runjags' package in R) in order to predict $F_{ub}$ at 100% plasma concentration. This model incorporated all 8 individual analytical measurements and accounted for analytical precision and calibration.

• Median $F_{ub}$ predictions from the JAGS model was compared against average quantitated $F_{ub}$ values based on duplicate trials for ~50 previously mentioned chemicals. Figure 6 shows that the JAGS model does a pretty good job of predicting $F_{ub}$ values ($R^2$ ~0.81), which indicates that we can reasonably trust the uncertainty estimates. Line edges represent the prediction error in the model; specifically the 5th and 95th percentiles.

## Conclusion

- For recent RED analyses of 85 ToxCast chemicals, a little over half (48) of their $F_{ub}$ values were quantitated at all three plasma concentrations plasma concentrations, which enables us to identify optimal RED assay methods for future studies.
- For 14 out of 34 chemicals whose $F_{ub}$ values were below limits of quantitation (LOQ) at 100% plasma concentration, their values could be determined at either 10% or 30% plasma concentration,
- The low correlation between high %-recovery and high $F_{ub}$ points to an opportunity to explore chemical binding, competition and/or degradation interactions that may take place inside of assay wells causing assay failure or underestimated readings.
- Bayesian analysis via JAGS proved useful in inferring $F_{ub}$ for environmental compounds. The results provided a quantitative estimate of measurement uncertainty in $F_{ub}$ that we can then use to estimate uncertainty in model predictions that use the measured Fub values.
- These new methods will increase the number of chemicals with available human equivalent doses (experimental or inferred) for further prioritization.

## References

1. Judson, R., Richard, A., Dix, D. J., Houck, K., Martin, M.,Kavlock, R., Dellarco, V., Henry, T., Holderman, T., Sayre, P., Tan, S., Carpenter, T. and Smith, E. (2008). The Toxicity Data Landscape for Environmental Chemicals. Environ Health Perspect 117(5), 685-695.
2. Bucher, J. R. (2008). Guest Editorial: NTP: New Initiatives, New Alignment. Environ Health Perspect 116(1).
3. Judson, R. S., Houck, K. A., Kavlock, R. J., Knudsen, T. B., Martin, M. T., Mortensen, H. M., Reif, D. M., Rotroff, D. M., Shah, I., Richard, A. M. and Dix, D. J. (2010). In Vitro Screening of Environmental Chemicals for Targeted Testing Prioritization: The ToxCast Project. Environmental Health Perspectives 118(4), 485-492.
4. Judson, R. S., Kavlock, R. J., Setzer, R. W., Cohen-Hubal, E. A., Martin, M. T., Knudsen, T. B., Houck, K. A., Thomas, R. S., Wetmore, B. A. and Dix, D. J. (2011). Estimating Toxicity-Related Biological Pathway Altering Doses for High-Throughput Chemical Risk Assessment. Chemical Research in Toxicology 24(4), 451-462. 10.1021/tx100428e.
5. Rotroff, D. M., Wetmore, B. A., Dix, D. J., Ferguson, S. S., Clewell, H. J., Houck, K. A., Lecluyse, E. L., Andersen, M. E., Judson, R. S., Smith, C. M., Sochaski, M. A., Kavlock, R. J., Boellmann, F., Martin, M. T., Reif, D. M., Wambaugh, J. F. and Thomas, R. S. (2010). Incorporating human dosimetry and exposure into high-throughput in vitro toxicity screening. Toxicological Sciences 117(2), 348- 58. 10.1093/toxsci/kfq220.
6. Wetmore, B. A., Wambaugh, J. F., Ferguson, S. S., Sochaski, M. A., Rotroff, D. M., Freeman, K., Clewell, H. J., 3rd, Dix, D. J., Andersen, M. E., Houck, K. A., Allen, B., Judson, R. S., Singh, R., Kavlock, R. J., Richard, A. M. and Thomas, R. S. (2012). Integration of dosimetry, exposure, and high- throughput screening data in chemical toxicity assessment. Toxicological sciences : an official journal of the Society of Toxicology 125(1), 157-74. 10.1093/toxsci/kfr254.
7. Waters, N. J., Jones, R., Williams, G. and Sohal, B. (2008). Validation of a rapid equilibrium dialysis approach for the measurement of plasma protein binding. Journal of Pharmaceutical Sciences 97(10), 4586-4595. 10.1002/jps.21317.



U.S. Environmental Protection Agency
Office of Research and Development