Mechanistic ADME Models: Qualifying Very Low Plasma Unbound Fractions

Hinnerk Boriss

Sovicell, Friedrich Ebert Str 16, 04109 Leipzig, Germany, hbo@sovicell.com

Mechanistic Evidence

Conventional ADME assays typically infer a biological parameter directly from an experimental endpoint. Mechanistic ADME Modeling (MAM) instead integrates informative experimental perturbations with assay-specific mechanistic models to infer underlying biological parameters and test whether the observed data support the assumptions required for their interpretation. MAM therefore asks not only what parameter value best explains the data, but also *whether the proposed biological mechanism adequately explains the experimental observations.*

Endpoint-Based Measurement of Plasma Free Fraction

For example, plasma protein binding is commonly measured by equilibrium dialysis, ultrafiltration, or ultracentrifugation. These methods are fundamentally endpoint-based: f_u is calculated from concentrations measured after physical separation of bound and unbound compound. By themselves, however, these measurements provide little internal evidence that the observed endpoint truly reflects the intended binding equilibrium rather than assay-specific effects. A plausible f_u value can therefore be obtained even when the assumptions required for its biological interpretation are not fully supported.

When Orthogonal Confirmation Is Not Independent Evidence

For very highly bound compounds, regulatory guidance allows measured f_u<0.01 when the reliability of the measurement is demonstrated; for novel or emerging methods, qualification against an established orthogonal method is expected. But agreement between two methods is not necessarily independent confirmation. Equilibrium dialysis, ultrafiltration, and ultracentrifugation differ experimentally, yet can share important liabilities such as nonspecific binding, incomplete or disturbed equilibrium, poor recovery, protein contamination or leakage, and limited analytical sensitivity. Shared failure modes can therefore produce agreement for the wrong reason, while method-specific artefacts can produce disagreement even when one result is valid. Orthogonal testing reduces uncertainty only when the second method provides genuinely independent evidence.

Adding Mechanistic Evidence

The TRANSIL Equilibrium Shift Assay provides an example of this approach. Rather than deriving f_u from a single separation endpoint, the assay generates a matrix of binding equilibria under systematically varied experimental conditions and analyzes all observations with a mechanistic binding model. The resulting f_u estimate is therefore accompanied by additional evidence from model–data agreement, residual structure, parameter identifiability, and parameter stability. Together, these tests ask whether the assumed binding mechanism and equilibrium behavior are consistent with the observations used to derive f_u.

The Equilibrium Shift Assay: Generating Mechanistic Evidence

The TRANSIL Equilibrium Shift Assay is designed to test a binding model, not simply to generate an additional f_u endpoint. A test compound is exposed to systematically varied combinations of plasma dilution and albumin-coated beads, creating a matrix of distinct competitive binding equilibria. All observations are analyzed simultaneously using an explicit mechanistic model of binding to soluble plasma proteins and the immobilized binding phase. Because a single parameter set must explain the complete experimental matrix, the experiment provides multiple internal consistency checks on the assumed binding mechanism. Model–data agreement, residual structure, parameter identifiability, and parameter stability therefore provide evidence for whether the resulting f_u estimate is mechanistically supported.

One f_u estimate tested across 25 experimentally distinct equilibria

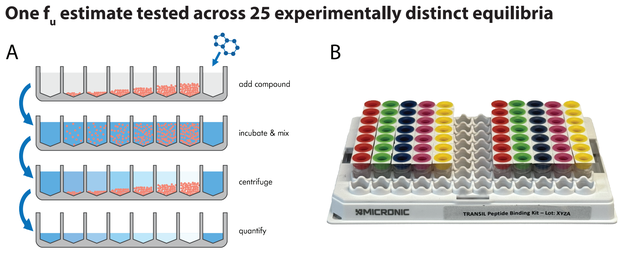


Fig. 1: A: Illustration of the assay workflow. The test item is added at a constant concentration to all wells containing plasma and varying amounts of beads. Incubation is carried out with 120 aspiration–suspension cycles to ensure equilibrium. Following centrifugation, the beads are pelleted and the supernatants collected for LC–MS/MS analysis of compound concentrations.
B: picture of the assay kit in 96 well format for testing two compounds using 5 plasma dilutions.

MAM provides internal validation of plasma binding results by testing whether the assay behaves mechanistically as expected

Model-based plasma protein binding study

A test item was analyzed across five plasma species using the TRANSIL Equilibrium Shift Assay. The assay combines plasma dilution with titration against human-albumin-coated beads and uses a mechanistic model to estimate species-specific plasma binding and free fraction f_u. Internal validation is provided by residual fit metrics and by the consistency of bead binding across plasma species.

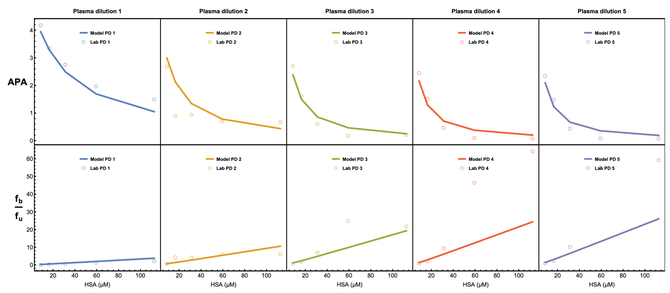


Fig. 2: Observed laboratory data are shown as open circles and the global model fit as solid lines. The upper row shows APA values, representing test item remaining in the supernatant. The lower row shows f_b/f_u, the ratio of bead-bound to unbound compound. Each column represents one plasma dilution, from highest plasma concentration (PD 1) to lowest (PD 5). Across dilutions, increasing bead HSA reduces APA and increases f_b/f_u, consistent with transfer of test item from supernatant to bead phase. Agreement between data and model supports internal consistency of the ESA fit.

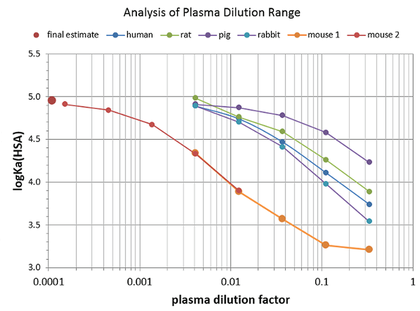


Fig. 3: Plasma dilution range analysis as internal model validation. Increasing plasma concentration progressively reduces the apparent bead affinity because soluble plasma proteins compete with the albumin-coated beads for the test item. Agreement between the measured dilution-dependent estimates and the full model supports the mechanistic consistency of the ESA measurement and strengthens confidence in the fitted plasma binding parameters.

Tab. 1: Species-specific plasma binding and model quality summary for the test item. The table provides an internal validation framework for the ESA result. Species-dependent f_u values are interpreted together with residual fit metrics and bead K_D consistency. Stable bead K_D across species confirms that the constant human-albumin bead phase behaves as an internal reference, while acceptable nRMSE values support the reliability of the global model fit.

Species	KD	fu	SSR	df	RMSE	nRMSE	N	KD(beads)	Fold range
Human	9,316 µM	1,5289%	2,5445	23	0,333 µM ✓	0,067	25	3,553 µM ✓	0,95
Cyno	13,022 µM	2,1243%	2,5772	23	0,335 µM ✓	0,067	25	3,455 µM ✓	0,93
Dog	19,495 µM	3,1469%	3,0311	23	0,363 µM ○	0,073	25	4,049 µM ✓	1,09
Rabbit	11,834 µM	1,9342%	4,6071	23	0,448 µM ○	0,090	25	4,168 µM ✓	1,12
Rat	69,877 µM	10,4313%	2,3852	23	0,322 µM ✓	0,064	25	3,166 µM ✓	0,85
Mouse	142,890 µM	19,2343%	1,4921	23	0,255 µM ✓	0,051	25	4,115 µM ✓	1,10

Internal validation of model-based ESA results

Together, these analyses show that the ESA result is supported by multiple internal consistency checks. The APA and f_b/f_u plots demonstrate that the global model reproduces the measured bead-titration behavior across plasma dilutions. The plasma dilution range analysis confirms the expected mechanistic shift from bead-dominated to plasma-dominated binding. The summary table integrates the biological readout with fit-quality and bead-consistency metrics, showing that species-dependent f_u differences are supported by acceptable residuals and stable bead K_D. Overall, the data support reliable model-based estimation of species-dependent plasma protein binding.

The Model: Testing the Binding Hypothesis

The mechanistic model describes the distribution of compound between soluble plasma proteins, the immobilized albumin phase, and the unbound state. A single set of binding parameters is fitted globally to the complete equilibrium-shift dataset and used to derive f_u. Importantly, the model is not used only to calculate the parameter. It represents an explicit hypothesis about the underlying binding process that must explain the experimental response across all plasma dilutions and bead concentrations. Model–data agreement, residual structure, parameter identifiability, and parameter stability therefore provide complementary evidence for whether the inferred f_u is supported by the observed binding behavior.

The model describes reversible binding of the test compound to soluble albumin and AGP and to immobilized albumin on the TRANSIL beads. A single set of binding parameters must explain all experimental equilibria simultaneously. Deviations between predicted and observed behavior therefore provide direct evidence when assumptions about binding, equilibration, or assay setup are inconsistent with the data.

The model converts the assay from an endpoint measurement into a testable mechanistic hypothesis

Conclusion:
Internal validation by model-data agreement

(1) For each plasma species, the Equilibrium Shift Assay generates one f_u estimate from 25 experimentally distinct equilibria.

(2) Agreement between APA data, f_b/f_u behavior, and the full model provides internal validation of the result.

(3) Stable bead K_D across species supports the use of the bead phase as an internal reference control.

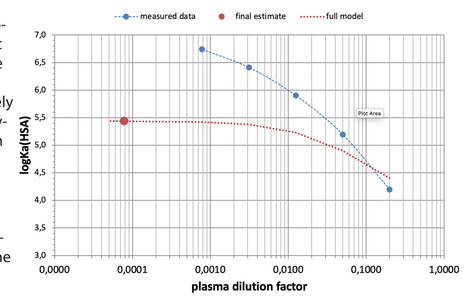
(4) Species-dependent f_u estimates are therefore supported by both fit quality and mechanistic consistency.

(5) Model-data diagnostics can flag compromised ESA results before fitted K_D or f_u values are overinterpreted.

(6)This enables both confident reporting of valid datasets and early rejection or troubleshooting of compromised experiments.

A reliable ESA result is supported when APA, f_b/f_u, dilution response, residuals, and bead K_D are all consistent with the same mechanistic model.

Fig. 4: QC detection of ESA model-assumption failure. Measured dilution-dependent apparent bead affinity does not follow the full ESA model, indicating loss of internal consistency across plasma dilutions. A likely cause is plasma-dilution-dependent recovery loss, where reduced protein content in highly diluted plasma allows sticky or poorly soluble test item to precipitate, adsorb nonspecifically, or show reduced analytical recovery. This unmodeled loss can mimic bead binding and prevents reliable estimation of plasma binding from the full dilution series.



Value of the mechanistic model in a failed experiment

This failed experiment illustrates why mechanistic assay modeling is more than curve fitting. The ESA model does not only estimate plasma binding; it also tests whether the data follow the expected equilibrium shift between soluble plasma binding and bead binding. In this case, the measured dilution-dependent apparent bead affinities do not follow the full model, indicating that the assay result is not internally consistent across plasma dilutions.
This pattern suggests that an additional, unmodeled process affects the measurement. For sticky or poorly soluble compounds, plasma proteins may stabilize the test item in solution. At high plasma dilution, reduced protein content can lead to precipitation, nonspecific adsorption, or reduced analytical recovery. The model flags this because the resulting signal cannot be explained by equilibrium redistribution alone.

The Bigger Picture

Mechanistic modeling has become an integral part of drug development because it does more than generate predictions: it makes assumptions explicit and allows their adequacy to be tested against experimental and clinical observations. PBPK applies this principle to drug exposure, while QSP extends it to biological and pharmacological response. MAM brings the same logic upstream to the experimental ADME parameters that feed these models, using mechanistic assays to infer and qualify the parameters before they are propagated downstream. The practical implication is straightforward: sophisticated models cannot compensate for poorly supported inputs. Better-qualified ADME parameters provide a stronger foundation for better predictions.

A three-layer framework for mechanistic drug modeling

