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Moderate to high throughput *in vitro* binding kinetics for drug discovery

Rumin Zhang[1], Christopher M. Barbieri[1], Margarita Garcia-Calvo[1], Robert W. Myers[1], David McLaren[1], Michael Kavana[1]

[1]In vitro Pharmacology, Merck Research Laboratories, 2015 Galloping Hill Road, Kenilworth, New Jersey, USA

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1. ABSTRACT

This review provides a concise summary for state of the art, moderate to high throughput *in vitro* technologies being employed to study drug-target binding kinetics. These technologies cover a wide kinetic timescale spanning up to nine orders of magnitude from milliseconds to days. Automated stopped flow measures transient and (pre)steady state kinetics from milliseconds to seconds. For seconds to hours timescale kinetics we discuss surface plasmon resonance-based biosensor, global progress curve analysis for high throughput kinetic profiling of enzyme inhibitors and activators, and filtration plate-based radioligand or fluorescent binding assays for receptor binding kinetics. Jump dilution after pre-incubation is the preferred method for very slow kinetics lasting for days. The basic principles, best practices and simulated data for these technologies are described. Finally, the application of a universal label-free technology, liquid chromatography coupled tandem mass spectrometry (LC/MS/MS), is briefly reviewed. Select literature references are highlighted for in-depth understanding. A new reality is dawning wherein binding kinetics is an integral and routine part of mechanism of action elucidation and translational, quantitative pharmacology for drug discovery.

2. INTRODUCTION

Binding kinetics (BK) is a universal molecular phenomenon for all biochemical processes. Thus, all metabolic pathways are mediated by enzymatic turnovers that occur with the initial substrate binding to and final product being released from the enzyme. Similarly, all regulatory mechanisms are accomplished by the binding of a regulatory molecule to its effector molecule and consequent conformational changes. Finally, all signal transduction pathways are a cascade of obligatory binding events beginning with the initial interaction of the signaling molecule with its receptor. Proteins in particular perform a host of biological functions, serving for example as enzymes, receptors, transporters, regulatory and signal transduction components, defense mechanisms, and structural, contractile and motor elements. Protein function is in turn controlled in response to various stimuli by the binding of their natural ligands or substrates, as well as by allosteric ligands that modulate their activity. Such ligands can be small molecules, peptides, proteins or nucleic acids. Importantly, the actions of these native ligands can be phenocopied and improved upon by synthetic molecules (pharmacological agents) that also act as orthosteric and allosteric inhibitors and activators of their targets. Defining the parameters that comprise protein/ligand interactions is central to our understanding of both modern biochemistry and the drug discovery process.

All drugs manifest their efficacy and toxicity by binding to “appropriate” and “inappropriate” targets with various levels of occupancy and duration. The manner in which binding kinetics can fine tune pharmacodynamics has recently been recognized (1-5), most notably using the concept of drug-target residence time (6-13). Binding kinetics encompasses not only molecular

Table 1. Summary of prevailing technologies for kinetics in drug discovery

Kinetic timescale	Prevailing method	Key advantages	Key requirement
Nanoseconds	Time-resolved fluorescence	Dynamics/binding/function based ultrafast kinetics	Fluorescence reflecting the rapid kinetics
Milliseconds to seconds	Stopped or quenched flow	Binding/function based fast kinetics, low to moderate throughput	Specialized instrument
Seconds to hours	Surface plasmon resonance or other biosensors	Binding based moderately fast to slow kinetics, moderate throughput	Specialized instrument
	Global progress curve analysis	Binding/function based moderately fast to slow kinetics, high throughput	Continuous detection preferred, though not obligatory
	Radioligand or fluorescent ligand binding assay	Binding based moderately fast to slow kinetics, high throughput	Radiolabeling or fluorescent labeling
Hours to days	Jump dilution	Binding/function based very slow kinetics, moderate to high throughput	Long stability of complex

association and dissociation; it also broadly includes reversible isomerization via conformational dynamics and irreversible inactivation via catalysis, aggregation or precipitation. Binding kinetics, together with enzyme turnover kinetics, provides a quantitative description and mechanism of action (MOA) understanding for all pathways in any biological system. Multiparametric models are known to exhibit both "sloppiness" and "stiffness", i.e., insensitivity and sensitivity, respectively, of model response to parameter changes (14-18). Critical nodes of interactions exist for the complex web of an interactome (19-22) and complementary experimental design can help to determine most or all parameters reliably (14, 18). Identifying such critical interaction nodes and quantitatively measuring the associated kinetics by *in vitro* and *in vivo* methods to predict model responses constitutes an essential task for systems biology and systems pharmacology.

A number of *in vitro* technologies are available for measuring kinetics on the various timescales encountered in biomedical research and drug discovery (Table 1). For ultrafast, nanosecond timescale kinetics, time-resolved fluorescence is used to monitor protein folding and conformational dynamics as well as for ultrafast enzyme reactions. This ultrafast technology is out of the scope of the present review and may be elaborated in a future update of this review. For fast, millisecond to second timescale kinetics, stopped flow and quenched flow are used to monitor transient and (pre)steady state binding and catalytic events. We will focus on the automated stopped flow method. For ordinary second to hour timescale kinetics, biosensors of various detection schemes such as surface plasmon resonance (SPR) (23, 24), SPR imaging (25-28), biolayer interferometry (29), back scattering interferometry (30-32), resonant waveguide grating and dual polarization interferometry (33), plasmonic-based electrochemical impedance imaging (34), DNA-based switchSENSE (35-38), and second harmonic generation imaging (39) are used to monitor biomolecular interactions and, for some platforms, also conformational dynamics. Biosensor methods complement traditional radioligand binding assays performed in the scintillation proximity or microtiter plate filtration assay formats. High throughput kinetic profiling of enzyme inhibitors/activators or receptor antagonists/agonists by global progress curve analysis has been developed (40, 41). The kinetic size-exclusion chromatography method is able to monitor kinetics on the timescale of seconds to minutes (42, 43). For slow timescale kinetics that takes hours to days, preincubation followed by jump dilution is used to monitor the very slow dissociation of a binary complex (44). Finally, liquid chromatography coupled tandem mass spectrometry (LC/MS/MS) can be used in conjunction with many of the above techniques.

In this review, we outline the basic operating principles and key advantages and requirements of six prevailing technologies commonly used in our laboratories for moderate to high throughput *in vitro* kinetics studies in drug discovery. We also share the best practices for each technology. We illustrate the timescale and observation of each technology with simulated data. Readers interested in other *in vitro* and *in vivo* technology platforms (such as calorimetry-based kinetics (45-47), single molecule kinetics (48-53) or positron emission tomography based kinetics (54-60)) should consult the references cited herein and elsewhere.

3. *IN VITRO* TECHNOLOGIES FOR BINDING KINETICS

3.1. Automated stopped flow method

Stopped flow instrumentation for fast kinetics was developed more than 75 years ago (61, 62) and available for general use for more than 50 years (63). Until recently, it was not easily adaptable to high throughput data generation and analysis. The main difficulties

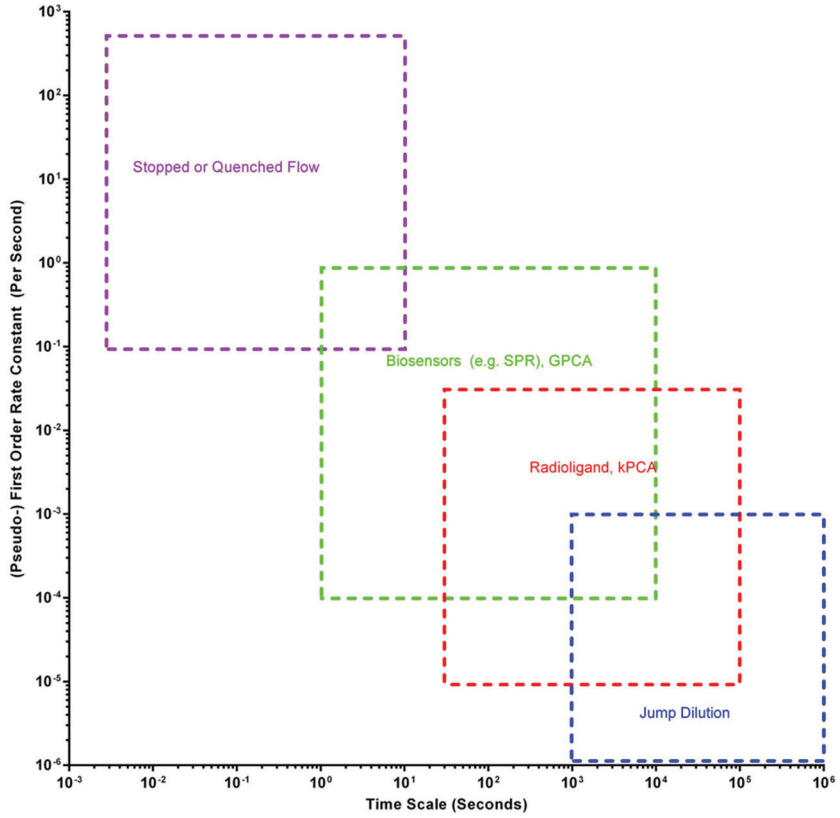

Figure 1. Timeframe coverage by the prevailing kinetic profiling technologies. The dotted square boxes indicate approximate timeframe of each technology and the corresponding first order or pseudo-first order rate constant that can be reliably measured. Liquid chromatography coupled tandem mass spectrometry (LC/MS/MS) can be used as a universal label-free detection method in conjunction with other technologies such as quenched flow, biosensors, global progress curve analysis (GPCA), unlabeled ligand binding (contrary to radioligand or time resolved fluorescence resonance energy transfer based "kinetic probe competition assay" or kPCA) and jump dilution. All together, these technologies cover up to nine orders of magnitude from milliseconds to days.

preventing adaptation to higher throughput screening included a tedious sample handling requirement for accurate use of the instrument, relatively large sample consumption, and difficulty of rapid data analysis. Recent applications of robotic automation for liquid handling coupled to automatic computational analysis with instruments such as the Auto SF-120 from KinTek Corp. are significantly enhancing the throughput of stopped flow data acquisition and analysis, providing the impetus for us to discuss this technique in the present review.

The design of stopped flow instruments has been described extensively elsewhere (61, 63-65). Briefly, a stopped flow apparatus consists of two or more loading syringes containing separate reactants and a stop syringe that rapidly stops the flow of the reactants in a detection chamber. Generally, the monitoring of millisecond (ms) timescale reactions requires rapid and sensitive detection normally achieved through optical methods (66), e.g. absorbance, fluorescence, static light scattering, or circular dichroism. Alternate detection methods such as NMR (67, 68), EPR (69), and small angle X-ray scattering (70) also have been used, but these are generally reserved for analysis of slower reactions. Most commercially available optical detection-based instrumentation has a dead time in the 0.5 – 2 ms range allowing for measurement of reactions with millisecond half-lives (See Figure 1). While the absolute limits of detection are predicated on the intensity of the optical signal change, under some circumstances association rate constants, k_{on}, on the order of 1×10^8 $M^{-1}s^{-1}$, can be reliably determined (64, 65).

The best practices for stopped flow assay design are listed in Table 2. For single turnover or transient enzyme kinetics, a sub-stoichiometric amount of substrate, compared to enzyme concentration, should be used. For (pre)steady state enzyme kinetics and simple binding kinetics, up to saturating level of substrate or ligand is used. For the simplest cases, the kinetics of the binding interaction between two reactants is measured directly by the difference in optical signature between the free and bound forms of the reactants (Figure 2A). In a 1-step binding interaction where one reactant is in large excess, this interaction follows a pseudo-first order exponential change. Kinetic

Table 2. Best practices for automated stopped flow method

Issues	Best practices
Design of mixing	Pseudo-first order for (pre) steady state kinetics
Stoichiometry of binding	Sub-stoichiometric ratio for transient, single turnover kinetics; excess for dose-dependent binding or turnover kinetics
Concentration	Adequate for signal/noise ratio (S/N >10)
Timescale of detection	Exponential, capturing >4 halftimes
Mode of detection	Fluorescence, fluorescence polarization, absorbance, light scattering
Temperature control	Thermostatic for reaction chamber and reagents
Data analysis	Concentration-dependent k_{obs} or amplitude; concentration-independent dissociation, isomerization or inactivation

constants can be characterized monitoring the change in k_{obs} (the apparent pseudo-first order rate constant for reaching steady state or equilibrium) that occurs as the concentration of one of the reactants is varied. Plotting the k_{obs} versus reactant concentration will yield a straight line with the slope indicating the k_{on} and the Y-intercept representing the k_{off}. A hyperbolic line would indicate a 2-step kinetic mechanism such as association before or after an isomerization step resulting in higher or lower asymptote, respectively (12). From this method of analysis, the value of k_{on} can be well determined while the value of k_{off} may be associated with large error since association and dissociation often occur within significantly different timescales. Accurate analysis of k_{off} is limited by the reactant concentrations required to enable detection, while still ensuring pseudo-first order kinetics of association. In practice this limits direct determination of k_{off}, and by extension K_d, to binding interactions with rapid dissociation (k_{off} > 0.01 s^{-1}) (64).

As noted above (Table 1 and Figure 1), stopped flow methodology provides a solution based method for monitoring reactions occurring at millisecond to second scales. Also notable, for interactions that are associated with a change in optical signal of sufficient magnitude for detection with this instrumentation, direct measurement of association of reactants can be made. This enables reactions in biological systems to be studied in complexities ranging from whole organisms (71) to purified proteins. Despite the broad range of systems that can be monitored by stopped flow there are certain limits to the technique that are not shared by the remaining methods discussed below. Due to the reliance on optical detection, interactions displaying nonspecific optical signals can interfere with detection. Also for most commercially available equipment, volumes approaching 100 µL are necessary for each reaction measured and in many cases, high concentrations of reactants (~µM) are required for a sufficient optical signature to be monitored.

While rapid kinetics can be monitored continuously in real time by stopped flow method, quenched flow method can also be employed to prepare effectively quenched fast reaction aliquots which are subsequently monitored discontinuously by a variety of offline detection schemes, most notably the universal, label-free mass spectrometry (see Section 3.6).

3.2. Surface plasmon resonance based biosensors

There are various platforms of biosensors as mentioned earlier. This review focuses on the most widely used surface plasmon resonance (SPR) based biosensors, such as BIAcore series of GE Healthcare and, more recently, Pioneer series of SensiQ Technologies employing Taylor dispersion to create a continuous concentration gradient spanning three orders of magnitude. The basic principle of SPR has been reviewed elsewhere (23, 24). Briefly, under total internal reflection conditions from a glass prism coated with gold film, the photons in incident light at a certain resonance angle will have the same momentum as the surface plasmons (i.e., free conduction electrons on the gold surface), resulting in resonant energy transfer from photons to plasmons and a concomitant loss of reflection intensity at this resonance angle. This surface plasmon resonance angle is sensitive to the reflective index changes within ~100 nm of the gold surface, such as on the adjacent carboxymethyl dextran layer where biomolecular interaction occurs. The SPR angular shifts as a function of the refractive index changes due to binding and mass increases form the basis of SPR detection. The detection area is usually a small fraction of a nanoliter on the biosensor chip. The detection is continuous, at a frequency of up to 40 Hertz for one interaction spot. This detection speed along with the dispersion at solvent change interface sets the upper or fast limit of kinetics to be < 1 s^{-1}. The lower or slow limit of kinetics depends on the baseline drift and is usually around 10^{-4} s^{-1}. The above two limits set the timescale of SPR-based binding kinetics on the orders of seconds to a few hours. The instrument setup for injection volume and flow rate usually allows association time lasting several minutes, which enables k_{on} to be measured reliably between ~10^3 and 10^7 (up to 10^8 for proteins) M^{-1} s^{-1}. Such k_{on} values, when coupled with the dynamic range of k_{off} (10^{-4} to 10^{-1} s^{-1}), allow SPR-measurable

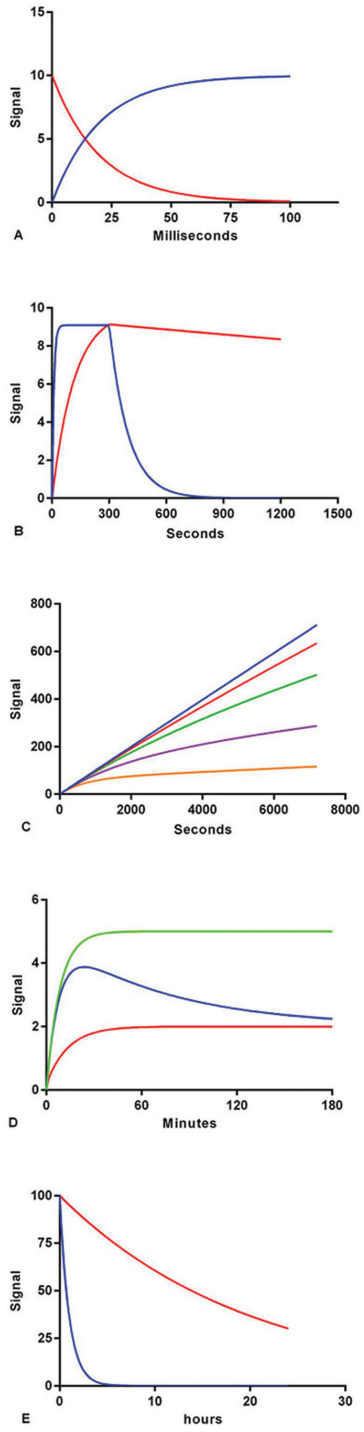

Figure 2. Simulated data for prevailing kinetics profiling technologies. (A) Stopped or quenched flow data for signal increase (blue) and decrease (red) fast kinetics under pseudo-first order condition; (B) Surface plasmon resonance (SPR) data for fast (blue) and slow (red) on-rate and off-rate binding kinetics; (C) Global progress curve analysis (GPCA) for time- and variously colored dose -dependent, 1-step slow binding enzyme inhibitor; (D) Radioligand, TR-FRET based kinetic probe competition assay (kPCA), or truly label-free ligand binding (e.g. by LC/MS/MS) data in the absence (green) or presence of faster (blue) or slower (red) dissociating competitor; (E) Jump dilution data for faster (blue) and slower (red) dissociating ligand.

affinity constants in the range of ~ 1 pM to ~0.1 mM. This range may be extended to < 1 pM with properly designed competition binding SPR.

The best practices for SPR-based binding kinetics are summarized in Table 3. More instructions and applications can be found in a book on SPR (72). Despite the diverse offering of biosensor chips, we recommend the nitrilotriacetic acid (NTA) derivatized chips and Streptavidin or NeutrAvidin (deglycosylated avidin) immobilized directly to CM chips by amine coupling or captured indirectly on CAP chips via DNA hybridization. This is because of the widespread use of generally non-perturbing histidine (e.g., decahistidine (73)) and biotin tags on recombinant proteins (74, 75), as well as the stability of the capture. The NTA surface can also be easily regenerated by 200 mM imidazole (which removes the His-tagged protein) or 350 mM EDTA (which removes both nickel and the His-tagged protein, and therefore nickel addition must precede protein capturing for next run). To prevent leaching, nickel NTA-captured His-tagged proteins may be further covalently, but sparingly, tethered to the matrix using very brief (seconds) and dilute amine coupling reagents. Covalent immobilization may inadvertently compromise the binding competence of the protein and should be used with caution. Streptavidin- or NeutrAvidin-coated chips may also be used to firmly capture biotinylated (preferably site-specific, mono-biotinylated) biomolecules, which gives no leaching due to the extremely high affinity binding and ultra-slow dissociation. Streptavidin indirectly captured via DNA hybridization provides not only stable immobilization of biotinylated proteins, but easy regeneration (such as with BIAcore biotin CAPture kit). Amine coupling under acidic conditions (pH 4-5.5), while universally applicable to untagged or non-biotinylated proteins, suffers from the potential of damaging some or all protein binding to some or all compounds. If amine coupling must be used, it is recommended to use a large molar excess of binding site-protective compound with K_d in sub-μM range and devoid of a reactive primary or secondary amine group under acidic condition that does not irreversibly denature the protein (as easily assessed by a thermal shift assay (76) in relevant buffers).

The level of immobilization should be as low as possible to allow final binding signals < 20 RU for small molecules and < 100 RU for large proteins binding kinetics. Preferably, biosensor chips with low density or thin matrix are used to minimize mass transport limitation and rebinding as well as surface heterogeneity (77). An exception to this rule is encountered when a high density of proteins on the biosensor surface is required to mimic the *in vivo* protein density at target site. Ideally, the immobilized protein should be homogenous, not prone to aggregation, equally binding-competent before and after immobilization and sufficiently stable to endure extended runs. The concentrations of analytes to be injected

Table 3. Best practices for surface plasmon resonance based biosensors

Issues	Best practices
Choice of biosensor chips	Nickel NTA or streptavidin/NeutrAvidin chip for capturing high-affinity and non-covalent His10-tagged or mono-biotinylated proteins, respectively
Level of immobilization	As low as detection limit permits and as high as *in vivo* target density dictates
Concentrations of analytes	Adequate to see apparent curvature in association for at least two sensorgrams
Multi-cycle or single cycle	Optimally designed single cycle is preferred, especially if regeneration is difficult
Dissociation length	Adequate dissociation (at least four halftimes unless very slow)
Regeneration	Use only when needed or shown to be non-harmful. Removal of His-tagged protein from NTA chip or biotinylated protein from CAP chip before re-capturing for subsequent cycle
Data analysis	Simple 1:1 binding model with mass transport limit, unless there is compelling reasons to use more complex models (such as heterogeneous analyte model for binding by racemic or otherwise competitive analytes)
Parameter quality control	Overall good fitting within limits, Chi-square <1%R_{max}, U (uniqueness) value <15, k_t (flow-rate corrected mass transport rate constant) >k_{on}*R_{max}

should be judiciously chosen before the run (definitely before an optimized re-run). This can be difficult without some prior knowledge of the binding kinetics. Future generations of SPR instrument should employ real time data analysis and automated decision making options in the experimental design to optimize the very first kinetic run and to minimize subsequent re-runs. For steady state affinity determination of fast-on and fast-off kinetics, analyte concentrations should bracket the steady state affinity constant. But for slow binding kinetics, one may have to use significantly more concentrated analytes to induce observable curvature during the association phase. Even with extended association at low flow rate (which may exacerbate mass transport limitation), steady state may not be readily achieved for slow binding analytes. As a rule of thumb, at least two well-curved sensorgrams in both association and dissociation phases are needed to permit reliable measurement of kinetic constants (78). The dissociation phase should be long enough to cover at least four halftimes, unless the dissociation is very slow and near the detection lower limit. In our experience, it is best to run ample buffer blanks throughout the runs, including in the beginning of the run to stabilize the baseline.

Biosensor surface regeneration is not needed for fast dissociating analytes. If needed with slow dissociation or irreversible binding, regeneration conditions for the NTA and CAP chips are the most straightforward, as mentioned earlier. Inclusion of a known allosteric compound that associates and dissociates rapidly (within seconds) and substantially enhances the dissociation of a slow binding analyte is useful for regeneration. Unless regeneration is demonstrated to have negligible effect on the immobilized protein and binding kinetics, repetitive regeneration should be minimized. One way of accomplishing this is by single-cycle kinetics, i.e. multiple, stacked up injections of increasing concentrations of analyte are followed by a single adequately long dissociation. Care must be taken with single-cycle multiple injections since each successive injection will have progressively less free protein to bind (thus the apparent R_{max} is dwindling). It is prudent to choose the concentrations so that at least two well-curved association phases are observed with good magnitude of signal increase (compared to noise).

The data analysis of SPR sensorgrams are performed by global curve fitting. The veracity of the kinetic constants depends on the quality of the raw data. Nonspecific binding to biosensor matrix should be insignificant to minimize distortion of kinetic data after reference sensorgram subtraction. Attention must be paid to the buffer blank runs' offset drift and chronologically close blanks should be used for referencing. Not all data contribute equally to the analysis. Spikes around injection junctions are best removed. Long stretches of data (such as a long drifting baseline after a rapid dissociation) which contribute little to, or worse, distort, the overall fitting should be cropped out. Well-curved sensorgrams whose curvatures are properly emphasized in the fitting permit reliable analysis. For very slow dissociating compounds, only association rate may be determined. For fast-on and fast-off compounds that give straight up and down changes in sensorgrams, only the steady state affinity constant may be determined. For extremely fast-on compounds or with high density protein immobilization, mass transport limitation may hinder the accurate determination of association and dissociation rate constants. For kinetics exhibiting heterogeneous, two or more sites binding (especially with amine coupling method of immobilization), the reliability of determination will decrease with more floating parameters, even if the proper heterogeneous ligand model is used for curve fitting. Antibody bivalence will cause avidity due to rebinding to the same antigenic site. Antigen multivalence will cause "sticky mouse trap" phenomenon due to

Table 4. Best practices for global progress curve analysis

Issues	Best practices
Continuous detection	Design a chromogenic, fluorogenic or mass detectable product (for signal increase) or substrate (for signal decrease)
Enzyme stability	Stable during assay timeframe, best >>2 hours
Concentrations of enzyme	As low as required by adequate signal and sustained steady state over the assay time course
Concentrations of substrate	Near K_m, or well above K_m (if competitive) or below K_m (if uncompetitive) to decrease apparent affinity of compound
Concentrations of compound	Adequate to generate >2 good curves, bracketing K_i and K_i^* values
Time of detection	Long enough to observe some good curvature and yet no-inhibitor control remains linear

repeated rebinding to adjacent sites by the same injected, "bipedal walking" antibody. Protein binding kinetics with racemic or otherwise competitive analytes require curve fitting using the heterogeneous analyte model.

The final fit lines should adequately describe the overall majority behavior of binding kinetics. Preferably, the residuals should be randomly distributed with chi-square no more than ~1% of the R_{max}. The kinetic constants should be non-correlated, yielding a uniqueness (U) value less than 15. Verify that the flow rate-corrected mass transport rate constant (k_t) is more, ideally far more, than the product of k_{on} and R_{max} such that mass transport limitation is considered insignificant. Shown in Figure 2B is simulated biosensor data for binding kinetics with fast (blue curve) and slow (red curve) on-rate and off-rate near the detection boundary by SPR.

3.3. Global progress curve analysis

There are two types of kinetics assays, exclusively binding-based (such as SPR and radioligand binding) or jointly binding and function-based. Global progress curve analysis (GPCA), while equally applicable to exclusively binding-based, time- and dose-dependent assay (see Section 3.4 and also (40)), here refers specifically to a jointly binding and function-based assay that monitors the time- and dose-dependent enzyme inhibition or activation in the presence of enzyme substrate(s) and a test compound. Any binding event that is observably slow compared to the enzyme turnover kinetics will exhibit time- and dose-dependence that reflects the slow binding kinetics. That is the foundation for GPCA. A book chapter has been devoted to the details of experimental design and data analysis of this method (41). We summarize a few key points for best practices in Table 4.

A continuous detection of unquenched reactions is preferred over discontinuous detection of effectively quenched reaction aliquots, but sufficiently time-resolved data from discontinuous detection will permit reliable analysis. A continuous detection is best achieved with a signal increase assay monitoring chromogenic or fluorogenic product formation, but a signal decrease assay monitoring the disappearance of substrate is acceptable. Liquid chromatography coupled mass spectrometry (LCMS) can also be used for label-free detection of product and/or substrate. Another prerequisite for GPCA is the extended stability of enzyme over the time course of the monitored turnover reactions. The concentration of the enzyme should be low enough to allow for both reliable determination of high potency compounds under non-tight binding condition and sustained steady state during the assay time frame, but not too low to avoid enzyme sticking to assay wells or substantial dissociation of oligomeric enzyme. The concentration of substrate used is preferably near K_m value, especially when prior knowledge about the mode of compound binding (orthosteric or allosteric) is not available. Sometimes, higher concentration of substrate may be needed to sustain steady state condition or to weaken the competitive compound's apparent potency to avoid tight binding condition and allow reliable potency determination. Other times, lower substrate concentration, at the risk of shorter lived steady state turnover kinetics, may be used to weaken the uncompetitive compound's apparent potency.

Compound concentrations should bracket K_i and, for 2-step binders, also K_i^* to allow reliable determination of binding affinity. For slow binding compounds exhibiting $k_{off} < 10^{-3}$ s^{-1}, compound concentrations will have to be substantially higher than K_i and K_i^* to observe within a convenient time window the exponential curvature of time-dependent progress curves from initial to final steady state. As a result, the optimal concentrations of compound required for reliable determination of kinetic constants may not overlap with those required for reliable determination of binding affinity. To allow simultaneous determinations of both affinity and kinetics by GPCA, the compound concentrations may have to vary over a wide range. In addition, the time course must be adequately long to allow observable curvature from initial to final steady state for at least two compound doses. It is important to use an optimal combination of enzyme and substrate concentrations such that a sustained steady state in the absence of compound will be maintained.

The data analysis by global curve fitting of both time- and dose-dependent enzyme turnover data

Table 5. Best practices for radioligand binding assay

Issues	Best practices
Radiolabel	^{3}H, ^{14}C, ^{33}P, ^{35}S, ^{125}I, high specific activity, sufficient half life
Radioligand concentration	Adequate protein binding and signal
Radioligand binding affinity	Near protein concentration
Nonspecific binding ligand	Cold ligand chemically different from radioligand to minimize isotope dilution effect on the specific binding site
Protein concentration	As low as assay detection limit permits to avoid radioligand depletion ("tight binding" condition)
Sampling frequency	Adequate to cover >90% curvature (or 4 halftimes)
Dissociation after wash out or jump dilution	Add cold ligand to minimize rebinding

yield both affinity and k_{off} under optimal conditions for 1-step binders (e.g. Figure 2C). The k_{on} value can then be calculated. When k_{off} of a 1-step binder is too small (< 10^{-5} s^{-1}), only the association rate constant k_{on} (= k_{off}/K_i for inhibitors or k_{off}/K_d for activators) may be determined, unless with longer monitoring. When k_{off} of a 1-step binder is too large (> 10^{-2} s^{-1}), only K_i or K_d may be determined, unless with much faster monitoring (such as stopped flow). For 2-step binders, when the slow backward isomerization is too slow (k_6 < 10^{-5} s^{-1}), only K_i or K_d for the first and fast step may be determined, unless with longer monitoring. When k_6 is too large (> 10^{-2} s^{-1}), only K_i^* or K_d^* for the overall affinity may be determined, unless with much faster monitoring. We routinely use commercial software platforms such as GraphPad Prism and KinTek Explorer (79) for global curve fitting analysis.

3.4. Radioligand binding assay

Radioligand binding remains a preferred method for investigating the interaction of ligands with their target protein (for reviews see (80-82)). It is an extremely versatile technique that does not require costly instrumentation and can be performed as an automated assay with moderate or high throughput. The main advantage of this technique is that it can be applied to the study of not only soluble and purified proteins but also to membrane preparations, extracts or whole cells. While this section discusses radioligand-based binding assays, the general principle universally applies to all ligand binding assays with or without radioactive isotope or fluorescent label. For example, a kinetic probe competition assay (kPCA) based on time-resolved fluorescence resonance energy transfer (TR-FRET) detection has been developed for high throughput binding kinetics (40).

The tracer used in radioligand binding studies should allow accurate detection of low levels of bound ligand (Table 5). Small molecule ligands are most commonly labeled with ^{3}H and peptide ligands with ^{125}I, although other radioisotopes (^{14}C, ^{33}P and ^{35}S) are also employed. Tritiated ligands generally behave identically to their unlabeled counterparts, have long radiochemical half-lives (12.5 years) and specific activities in the range of 20 to 80 Ci/mmol (depending on the number of tritium atoms incorporated in the molecule). These properties allow sufficient signal to study binding interactions having nanomolar affinities. Iodinated ligands, on the other hand, have short radiochemical half-lives (60 days) and specific activities of around 2000 Ci/mmol. Caution needs to be taken regarding whether the incorporation of ^{125}I results in altered binding properties *vs*. non-iodinated analogs, unless the radioligand is used primarily in competition assay to determine binding kinetics for unlabeled compounds (see later). Due to its high specific activity, low receptor densities and ligand binding interactions having picomolar affinities can be experimentally detected with ^{125}I, ^{33}P and ^{35}S labeling (83, 84).

Radioligand binding studies require parallel incubations of radioligand with receptor alone (for total binding) and in the presence of sufficient unlabeled ligand (for nonspecific binding) to occupy all the specific (but not nonspecific) binding sites of available receptors. If possible, it is best practice to use a displacing agent that is structurally different from the radioligand (thus competing for only the specific binding sites) and not the cold version of the radioligand that not only eliminates specific binding but probably reduces nonspecific binding due to isotopic dilution. The non-specific binding accounts for binding of the ligand with a much lower affinity to other components of the receptor-containing preparation and/or binding to materials used in the experiment. Thus, specific binding, or binding to the receptor, is defined as the binding to the target that it is displaceable by a large excess (usually 100 times its K_d) of cold ligand.

Another important point to consider in setting up a radioligand binding assay is the choice of technique (vacuum filtration, dialysis, centrifugation, gel filtration or precipitation) for adequate separation of bound and free ligand. Vacuum filtration is the most widely used with membrane preparations and whole cells (85). The bound ligand is retained on glass-fiber filters and the free ligand passes through. Following initial filtration, the filters are usually rinsed with cold washing buffer to reduce non-specific binding to the filter and to improve the signal

to noise ratio. This method is very reproducible and quick. However, the removal of free ligand necessarily promotes dissociation of the ligand from the receptor, thus, this technique is not suitable for ligands with fast off-rates. Gel filtration method is one of the best choices for soluble and purified receptors and exhibits excellent recovery of protein-ligand complexes and also less opportunity for dissociation (86). In addition to this, there are homogeneous radioligand binding methods that do not involve washing steps or removal of free radioligand. One of the most often used is the Scintillation Proximity Assay (SPA). It is a mix and read bead-based assay, can be adapted to soluble targets as well as membrane-bound receptors and has a high throughput. Moreover, association and dissociation experiments can also be performed, and it is an excellent technology to determine fast on and off rates by cycle counting that may be difficult to obtain by other methods (87, 88).

In kinetic experiments, the binding of one or more concentrations of radioligand is monitored at different time points and analyzed to estimate association (k_{on}) and dissociation (k_{off}) rate constants. The simplest scenario is the determination of k_{on} and k_{off} of the radiolabeled compound (89, 90). The standard analyses of ligand binding experiments assume that the concentration of free radioligand is constant during the experiment, which means that most of the ligand remains free and only a small fraction (less than 10%) is bound even at equilibrium (pseudo-first order conditions). The first best practice is to use a low concentration of receptor ($<< K_d$), as low as permitted but still giving reliable detection, to avoid “tight binding” condition under which significant radioligand depletion would cause non-pseudo first order kinetics. Then one should design a concentration- and time-dependent matrix by using increasing concentrations of the radioligand (thus, achieving varying levels of receptor occupancy) and monitoring each binding reaction over time until >90% equilibrium is achieved. If this approach is adhered to, a traditional re-plot of the observed apparent rate constants (k_{obs}) toward achieving equilibrium (obtained by simple mono-exponential curve fitting to each kinetic curve, see Figure 2D, green curve) as a function of total (non-depleted) ligand concentrations should yield a straight line with a slope equal to k_{on} and a Y-intercept equal to k_{off}. More simply and robustly, a global curve fitting approach (similar in spirit to GPCA method in section 3.3) is highly recommended for simultaneous analysis of all concentration- and time-dependent curves to derive k_{on} and k_{off} directly. It is also possible to determine k_{on} in an association experiment when only a single concentration of radioligand is used but it requires an independent determination of the maximum number of binding sites, B_{max}, present in the experiment. In any case, if the pseudo-first order conditions are incorrectly assumed, the value of k_{on} will be underestimated. The direct determination of the dissociation constant, $k_{off,}$ is less complicated. Once ligand and receptor binding have achieved equilibrium, the dissociation of the complex can be initiated by jump dilution into a solution containing a large excess of cold competing ligand (see section 3.5 for more details). The jump dilution and the excess competing ligand should be able to minimize rebinding of the dissociated labeled ligand. A failure to completely dissociate the ligand-receptor complex within a finite period of observation indicates the possibility of slow reversibility (slower than the observation time window), irreversibility, or allosteric binding. There should be good agreement of the K_d calculated in kinetic experiments with the K_d obtained in saturation equilibrium experiments if there is only one binding site, the binding is fully reversible and there are no tight binding or gross experimental errors. To alleviate tight binding for super high affinity compounds, one may consider a “double competition” setup where both a hot radioligand and a cold, structurally identical or different, competitor ligand of known affinity are used at proper concentrations to weaken the apparent binding affinities of compounds to be near or preferably well above the protein concentration while maintaining good radioligand binding signals.

Kinetic rate constants of unlabeled ligands can also be determined by radioligand binding competition, but they require prior determination of the association and dissociation rate constants for preferably an orthosterically competitive radioligand. A mathematical model was described (91) to determine the kinetics of binding of competitive ligands based on the experimental data of the binding kinetics of a dose-fixed radioligand in the presence of simultaneously added, dose-varied unlabeled ligand. In these studies, the presence of the unlabeled compound will alter the time course of the binding of the radioligand. If the competitor dissociates faster than the radioligand, the association of the radioligand will slowly and monotonically approach its equilibrium in time (see Figure 2D, red curve). If the competitor dissociates slower than the radioligand, the association curve will be biphasic and more radioligand will be bound at some intermediate time points than at equilibrium (see Figure 2D, blue curve).The two-step competition binding approach, or delayed association method, is another indirect method to estimate off-rates of unlabeled competitors (92). In this case, the receptor is preincubated with the competitive unlabeled ligand, the samples are washed off of free ligand, and subsequently the time course of the association of the radioligand is monitored. Radioligand binding can also be coupled to downstream functional readout to measure binding kinetics and drug-target residence time (93).

Competition binding kinetics may also be used to discriminate between allosteric and orthosteric modulators. In general, it is not always possible to determine if an inhibitory effect on radioligand association kinetics is allosteric or orthosteric except when an

Table 6. Best practices for jump dilution

Issues	Best practices
Preincubation stoichiometry, concentrations, time	Nearly stoichiometric, up to MicroM level, >30-min
Fold of jump dilution	>100-fold
Post-dilution condition	Apparent K_d or K_i >> (compound) ≈ (enzyme) > enzyme's low limit of detection
Minimizing rebinding	Use substrate near K_m or well above K_m (if competitive) or below K_m (if uncompetitive); use excess cold ligand
Monitoring time	Adequate to cover >90% dissociation (or four halftimes)
Monitoring method	Radioactivity, enzyme activity, MW of free ligand
Stability	Protein-ligand complex must be sufficiently stable over the course of monitoring

alteration of the dissociation rate of the radioligand (without the complication of rebinding) would be indicative of allosteric modulation (94). Similarly, a characteristic change of association rate constants in the presence of increasing levels of orthosteric or allosteric modulators is helpful to discern the mode of binding, since an orthosteric modulator will perpetually decrease the apparent k_{on} value while an allosteric modulator will either not change or asymptotically decrease or increase the apparent k_{on} values. As a result of the above changes to apparent k_{on} and k_{off} values, the apparent binding affinity changes of radioligand due to the presence of another allosteric or orthosteric ligand will also exhibit distinct patterns. Thus, an orthosteric competitor will perpetually weaken the radioligand affinity, while an allosteric modulator will either not change (by noncompetitor), or asymptotically increase (by uncompetitor) or asymptotically decrease (by allosteric competitor) the radioligand affinity. A monovalent ligand that orthosterically but partially competes with one of the two sites of a heterobivalent ligand may be mistaken as an allosteric modulator (95).

3.5. Jump dilution

To determine k_{off} of a very slow dissociating compound from its target, jump dilution followed by often discontinuous monitoring over the course of hours to days is the preferred method of choice. Jump dilution relies on the physical chemical principle that pre-established equilibrium, upon massive dilution (usually > 100-fold), will re-equilibrate to a new state. Thus, when a preformed compound/target complex is diluted massively into a solution to minimize re-binding between them, the compound will exhibit a first order dissociation kinetics (Figure 2E). There are several issues to pay attention to for jump dilution (Table 6).

The preformed complex between compound and its target protein should preferably be initiated with stoichiometric levels that reflect the true binding stoichiometry (1:1, 2:1, 1:2, etc). Preincubation using lopsided, non-stoichiometric levels of enzyme and tight binding inhibitor may suffer from diminished window of detection upon jump dilution (44). The concentrations during preincubation should be high enough (up to μM) to enable rapid equilibrium of binding between the compound and its target. The preincubation time should be adequately long to assure equilibrium binding. As a general rule of thumb for most compounds, preincubation at > 1 μM for > 30-min should be used. For very slow binding compounds, higher concentrations and longer time may be needed to achieve equilibrium binding. For 2-step binding inhibitors whose initial rapid binding is followed by a slow noncovalent isomerization or covalent modification, pre-incubation time is best varied for 30-min and many hours (up to overnight) to ascertain that reversible equilibrium or irreversible inactivation has been achieved.

The fold of jump dilution should be large enough to minimize compound rebinding, in conjunction with additional measures. It is best to dilute so massively that the final residual compound concentration is well below the apparent K_d or K_i and the final protein target concentration is still above low limit of detection (if enzyme turnover is used to monitor inhibitor/enzyme dissociation). For high affinity compounds (K_d or K_i < 1 nM), it may not be practical to dilute the compound concentration below its affinity constant and may require additional measures to decrease the *apparent* affinity and minimize rebinding. For enzymatic monitoring following jump dilution, high concentrations (>> K_m) of competitive substrate or low concentrations (<< K_m) of uncompetitive substrate can be used to decrease the apparent compound binding affinity so that the final residual compound is well below the *apparent* binding affinity. For non-enzymatic monitoring following jump dilution, it is best to use excess cold ligand (for radioligand monitoring) or orthosterically competitive ligand (for non-radioactive monitoring such as mass spectrometry). The monitoring time should preferably cover at least four halftimes unless the dissociation is extremely slow and the protein target stability is not sufficient to allow longer monitoring. For very slowly dissociating enzyme inhibitors, it is important to include a control reaction spiked with the same residual final compound concentration as achieved after jump dilution. This control reaction provides an accurate measure of

the final steady state velocity after full enzyme recovery from inhibition post jump dilution so that the exponential fitting will have the proper constraint to the final state.

For protein target that associates to form dimer, trimer or higher order oligomer, jump dilution may be complicated if there is rapid, significant dissociation of the protein target oligomer. The final protein concentration needed to maintain the oligomeric state may create a very tight binding condition for a high affinity ligand such that jump dilution experiment becomes impractical. In this case, alternative methods of binding kinetics will have to be considered.

3.6. LC/MS/MS

Traditionally, quantitation of bound and/or free ligand in binding studies was performed by radiolabeling the ligand of interest to a known specific activity with one of several biomolecule-compatible radioisotopes including ^{3}H, ^{14}C, ^{35}S, ^{33}P or ^{125}I. Radiolabeled ligands, in combination with modern radiochemical detection methods, allow for ligand detection in the pM to fM range (for details, see Section 3.4). While extremely valuable, radiochemical binding assays are limited by the need to successfully prepare a suitable radioligand for each compound of interest, the necessity of working with hazardous radioactive material and the expense associated with the resultant waste. The magnitude of these issues increases with the number of ligands of interest. Fluorescently labeled ligands have been used as an alternative, but the dye substituent is often bulky and hydrophobic, which can alter ligand binding properties (see for example (96)), unless such labeled ligands are used primarily in competition binding assays to examine the binding of unlabeled compounds.

A method in which bound and/or free ligand could be quantitated with comparable sensitivity in a label-free manner would be clearly advantageous. Analyte quantitation using liquid chromatography coupled tandem mass spectrometry (LC/MS/MS) on a triple quadrupole mass spectrometer (QqQ-MS) solves this problem. This section reviews this universal, label-free detection method and its applications to binding kinetics in drug discovery. In retrospect, this is a clear extension of the widespread use of LC/MS/MS to study the ADME properties of drug candidates (see for example (97)). LC/MS/MS offers unparalleled specificity, sensitivity and speed for analyte determination in complex mixtures. The key to this method is the combination of a high resolution liquid chromatography system (generally operated using reverse phase conditions) coupled in line to a QqQ-MS. The system, when used in selected (or multiple) reaction monitoring mode, allows the specific ligand of interest (and an internal standard) to be identified and quantitated with extremely high specificity and sensitivity using a unique combination of three parameters: (1) LC retention time; (2) Parent ion m/z peak (selected in quadrupole 1) and (3) A major fragment ion m/z (selected in quadrupole 3). What follows is a brief review of publications describing the application of LC/MS/MS to evaluate the binding of specific ligands to their receptor targets. A discussion of high throughput screening applications of MS to identify and characterize (unknown) ligands obtained by affinity selection methods is beyond the scope of this discussion (98), as is the characterization of non-covalent receptor/ligand complexes by electrospray ionization MS (99) and the determination of *in vivo* receptor occupancy by LC/MS/MS (100).

Application of LC/MS/MS analysis to ligand binding studies was first reported in 2003 (101). In this report, LC/MS/MS was employed to develop a competition binding assay for membrane bound dopamine D1 receptor utilizing the SCH 23390 ligand at ~ K_d levels (0.5 nM) in combination with LC/MS/MS compatible buffers. The increase in free SCH 23390 as a consequence of increasing competitor concentration was monitored by LC/MS/MS following removal of the receptor/ligand complex by ultracentrifugation; LC/MS/MS and radiochemical competition assay derived K_i values were in excellent agreement. This early protocol was improved in subsequent papers from the Wanner laboratory. One study used the dopamine D2 receptor and spiperone as the probe ligand and employed solid phase extraction to remove non-volatile binding buffer components and reduce matrix effects; haloperidol was incorporated as an internal standard to compensate for process variability and non-quantitative analyte recovery (102). A subsequent study, using the membrane bound GABA transporter mGAT1 and its high affinity ligand NO 711, described the LC/MS/MS-based binding protocol used most widely today, which incorporates the following modifications: (1) Per-deuterated NO 711 was used as a heavy atom labeled internal standard; (2) 96-well glass fiber filter plates in combination with a vacuum manifold were used to isolate transporter bound from free ligand; (3) Wash steps were employed to eliminate all free ligand and interfering buffer components from the protein/ligand complex entrapped on the filter; and (4) Bound ligand was liberated from its receptor by drying the plate, followed by protein denaturation and ligand elution with an organic solvent (typically methanol) at concentrations consistent with direct injection onto the LC/MS/MS (103). In this seminal publication, the authors first demonstrated the application of MS binding technology to measure k_{on} and k_{off} values, K_d and B_{max}, and also developed and utilized a competition MS binding assay to define ligand structure activity relationships (SAR). NO 711 binding to mGAT1 under more physiological conditions was also described, demonstrating the applicability of the improved LC/MS/MS binding protocol to lower affinity (K_d = 150 nM) ligands (104). The Wanner laboratory also focused on increasing the throughput of MS based binding experiments by utilizing MALDI/MS/MS (105)

Table 7. Best practices for LC/MS/MS

Issues	Best practices
Quantitation	Isotope-dilution using stable-labeled internal standard (where available); external standard curve prepared in sample matrix
Sample processing/instrumental variation	Include an appropriate stable-labeled or chemical analog internal standard
Post-processing sample stability	Maintain samples in a temperature-controlled autosampler; evaluate and document stability over expected analysis time
Ruggedness	Consider clean-up strategies for complex sample matrices (liquid-liquid or solid-phase extraction); make use of integrated instrument fluidics to divert LC flow to waste during void volume elution and column cleaning/regeneration portions of gradient
Limit of detection	Optimize ionization mode (negative vs positive ESI; APCI and APPI); low-flow enhances electrospray ionization
ESI: Electrospray ionization, APCI: Atmospheric pressure chemical ionization, APPI: Atmospheric pressure photoionization	

and by employing shorter LC columns/run times during LC/MS/MS analysis (106). Additional studies exploited increased incubation volumes to lower target concentration while maintaining sufficient bound ligand for LC/MS/MS detection; this maneuver can be used to measure high affinity interactions while avoiding the "tight binding" conditions (107). This paper is also notable in that it describes the use of a single ligand to probe three distinct monoamine transporters. An alternative approach to multiplexed assays employing LC/MS/MS analysis (several ligands/receptors in a single incubation) has also been described (108).

The application of LC/MS/MS methods to study ligand binding to soluble proteins is less well described. At least three publications describe the development and utilization of competition binding assays in which unbound ligand was quantitated following removal of the enzyme/ligand complexes by ultrafiltration (109-111). In our laboratory, we generally employ an alternative method in which the protein/ligand complex is isolated from free ligand using 96-well plates containing size exclusion chromatography media. The protein/ligand complex in the eluate is then dissociated by addition of organic solvent and analyzed by LC/MS/MS. We have used this approach to measure k_{on} and k_{off} values, K_d and B_{max}, and also for competition MS binding assay to define SAR for soluble receptor orthosteric and allosteric ligands. An alternative is to precipitate the protein/ligand complex (using PEG or ammonium sulfate) and isolate/wash the complex using 96-well glass fiber filter plates, similar to the popular method used for membrane-associated proteins (103).

Though highlighted here mostly for its application to equilibrium binding studies, LC/MS/MS has broad utility as a detection method for experiments designed to explore binding kinetics. Table 7 summarizes some best practices that should be incorporated into any experimental design employing this technique. First and foremost, the inclusion of an appropriate internal standard as early as possible in the sample preparation process is indispensable as a means to correct for sample to sample variation; it also serves as a useful tool during troubleshooting exercises. Where available, stable isotope labeled (^{2}H, ^{13}C or^{15} N) internal standards are preferred, but in their absence a suitable chemical analogue of the ligand being studied is often sufficient. If samples are to be prepared and stored prior to analysis or if the analytical runtime is long, it is wise to investigate sample stability under autosampler conditions. This is easily done by preparing a small number of replicate samples at relevant concentrations along with a suitable standard curve; these samples are analyzed at time zero and then maintained in the autosampler and re-injected along with a fresh standard curve at a subsequent time point to bracket the expected timeframe for storage or analysis. Acceptable agreement in the measured concentrations will establish post-processing stability. Samples of high complexity such as crude cell lysates and membrane preparations washed with detergents will contain matrix elements that can build up over time and contribute to deteriorating performance of the mass spectrometer. Simple strategies to mitigate the impact of this issue include the aforementioned internal standard, additional sample clean up prior to LC/MS/MS analysis (via solid phase or liquid-liquid extraction to isolate the analyte from undesired matrix elements) and the use of flow-switching fluidics to direct only the portion of the chromatogram where the analyte is known to elute to the mass spectrometer and to divert the remainder of the LC cycle to waste. Finally, it is inevitable that the need will arise to push the limit of detection as low as possible. In these cases, rigorous tuning of conditions affecting analyte ionization and fragmentation by an experienced mass spectrometrist will be helpful in achieving the optimal signal to noise ratio. This, coupled with advancements in mass spectrometers and the application of micro- and nanoflow LC, has made sub-nanomolar limits of detection increasingly common.

In summary, the application of LC/MS/

MS methodology provides an analytical method that enables the analysis of both equilibrium and kinetic binding parameters of virtually any ligand in a label-free manner to both membrane bound and soluble proteins with comparable sensitivity and specificity, but arguably increased experimental flexibility, versus radioligand or kPCA based binding approaches.

4. FUTURE DIRECTIONS

It has taken the scientific community quite some time to grapple with the necessity of understanding binding kinetics. Recognition of the importance of drug-target residence time, a celebrated component of binding kinetics, is now entering its second decade. With the enabling technologies for measuring *in vitro* binding kinetics covering timeframes over nine orders of magnitude as reviewed here, coupled with additional *in vitro* and *in vivo* kinetic profiling reported in literature, we anticipate a more intentional and proper use of these technologies by scientists in advancing their quantitative and translational pharmacology studies. One fundamental gap to achieving this goal is the lack of awareness regarding the pervasive role binding kinetics plays from everyday activity and binding assays to ultimate drug efficacy and safety. It has been demonstrated that residence time within the same series of chemical or biological molecules can be tunable, resulting in differentiated impact on efficacy and safety (112, 113). We encourage pharmacologists and the modeling and simulation community to actively incorporate binding kinetics into mechanistic and translational PKPD models (114, 115) and dynamically interact with experimentalists to fine tune their models. Modeling can also reciprocally benefit experimentalists by providing early identification of the critical binding network nodes and relevant kinetic parameters that will be essential to predict drug efficacy and safety. We anticipate that drug discovery will be increasingly grounded in quantitative systems biology and pharmacology and, as a result, yield a more bountiful medicine chest that efficaciously and safely tailors to the various medical needs of humanity.

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Abbreviations: Binding kinetics (BK), association rate constant (k_{on}), dissociation rate constant (k_{off}), mechanism of action (MOA), drug-target residence time, pharmacokinetics (PK), pharmacodynamics (PD), stopped flow, surface plasmon resonance (SPR), global progress curve analysis (GPCA), radioligand binding, Scintillation Proximity Assay (SPA), time-resolved fluorescence resonance energy transfer (TR-FRET) based kinetic probe competition assay (kPCA), jump dilution, liquid chromatography coupled tandem mass spectrometry (LC/MS/MS), drug discovery.

Key Words: Binding Kinetics, Bk, Stopped Flow, Surface Plasma Resonance, SPR, Global Progress Curve Analysis, GPCA, Radioligand Binding, Jump Dilution, Review

Send correspondence to: Rumin Zhang, *In vitro* Pharmacology, 2015 Galloping Hill Road, Kenilworth, NJ 07033, USA, Tel: 908-740-3430, Fax: 908-740-2207, E-mail: rumin.zhang@merck.com