

Parallel Line Analysis with Agilent BioTek Gen5 Data Analysis Software

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Abstract

The estimation of relative potency in biological assays is important in pharmaceutical drug development and toxicological quality control. In complex biological systems, a direct determination of potency of an agent cannot be made. Relative potency can be determined by the comparison of dose responses elicited from a known compound and the experimental compound. This application note describes the calculation of relative potency and parallel line analysis using Agilent BioTek Gen5 data analysis software.

Introduction

The comparison of dose-response curves plays an important role in many biological applications. One of the most commonly used applications is the comparison of the potency of different compounds. This is true with bioassays where there is no direct measurement of a product, but rather an effect is measured. For a meaningful comparison of the potency of different biological agents, the curves need to be parallel to one another when plotted on the same axis. The only difference would be a shift either right or left on the X-axis. Interpolation of the curves being compared at any Y-value would be indicative of the relative potency of the unknown as compared to the known agent (Figure 1). This methodology works well for linear regression analysis, where the slope of the line is unchanging across the concentration range. However, with four- and five-parameter analysis, the dose response is not the same over the entire tested concentration range. In these types of analysis, the response is a sigmoidal shaped curve with relatively little changes in response at both the highest and lowest concentrations. The response changes, relative to the concentration, only in the center concentrations. The point at which the curves are interpolated is important for relative potency determinations to be made. Typically, these comparisons are made at the 50% effective dose or ED_{50} .

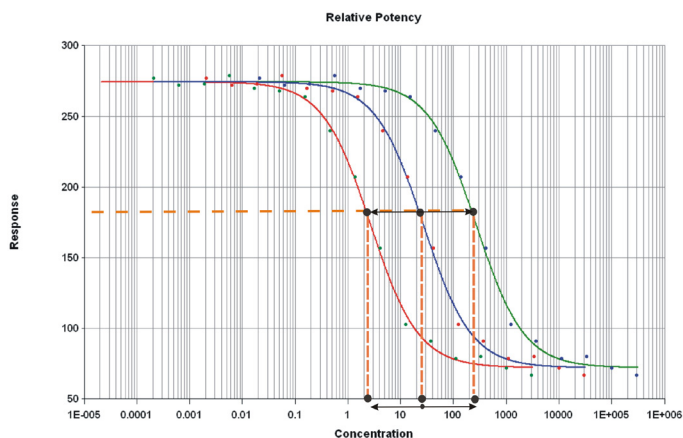


Figure 1. Illustration of relative potency. Reference (blue) and experimental dose-response curves (red and green) are plotted using constrained four-parameter logistic curve parameters. Relative potency is calculated from the change in interpolated concentration.

The difficulty of calculating relative potency is that biological systems rarely behave exactly as expected. Small experimental differences between samples result in curves that are not truly parallel. The results are curves that have the same maximum and minimum responses, but different slopes; or curves that have the same slopes, but different maximum and minimum responses.

Because relative potency calculations are difficult, if not impossible, when the curves are not parallel, Gen5 data analysis software uses a constrained model. In this model, the parameters describing the reference and experimental curves are calculated together, so that the parameters for curves are constrained to be the same for both curves. When nonlinear functions such as 4-P and 5-P fits are used, the minimum and maximum response parameters are also constrained to be the same for both curves. Using constrained curves results in an extra parameter, called relative potency, that describes the difference between the experimental curve and the reference curve. In this way, proper comparisons between the two dose-response curves can be made.

This application note describes the calculation of relative potency and parallel line analysis of an experimental and a reference compound measured for hERG binding with an Invitrogen kit. Data analysis was accomplished using Agilent BioTek Gen5 data analysis software.

Materials and methods

Predictor hERG Assay test kits were obtained from Invitrogen (Carlsbad, CA). The binding assay was carried out according to the kit instructions. Reagents were thawed and Predictor hERG membrane preparations were sonicated. Working 4x tracer was prepared by diluting the 250 nM stock provided in the test kit to 4 nM, with hERG FP assay buffer also provided. Dilutions of test compounds were prepared as 4x stocks from their final intended concentrations. Assays were performed in 384-well microplates. Aliquots (5 μ L) of each concentration of test compound were pipetted into the appropriate wells of the microplate. As required, 10 μ L of (2x) membrane preparation was then dispensed. Working Tracer solution (5 μ L) was then added, and the plate was allowed to incubate at room temperature for at least one hour prior to measuring fluorescence polarization. Fluorescence polarization measurements were made using an Agilent BioTek Synergy 2 multimode reader.

Measurements were made from the top using the tungsten light source. Both parallel and perpendicular fluorescence were measured using the same 530/25 excitation and a 590/35 emission filter, along with a 570 nm cutoff dichroic mirror. The PMT sensitivity was set automatically so that the positive control well had a raw fluorescence value for the parallel signal of 50,000 relative fluorescent units (RFU). Polarization values were calculated automatically using Agilent BioTek Gen5 data analysis software. In curve analysis, the astemizole curve was designated to be the reference curve, while the E_{410} was designated the sample curve for relative potency comparison to that of astemizole (Figure 2).

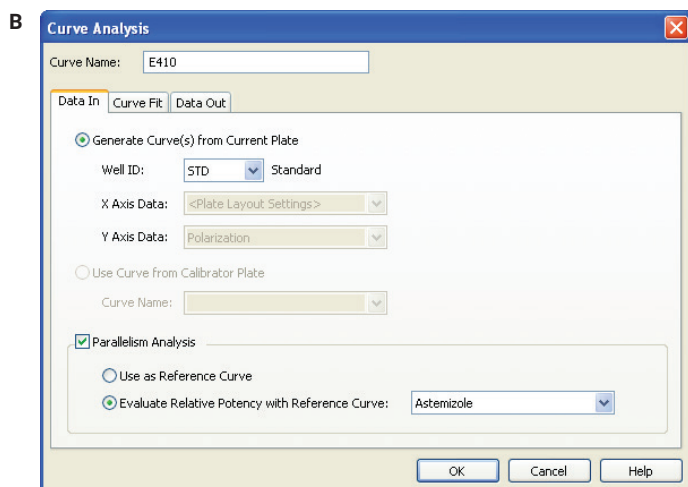
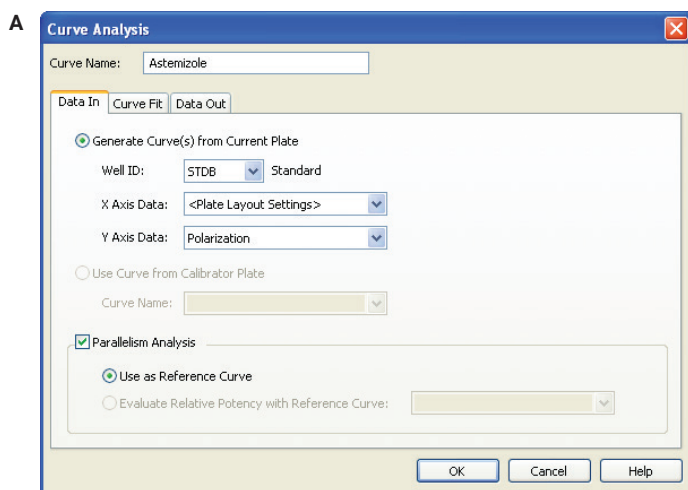


Figure 2. Parallelism analysis data reduction steps. The two curves are delineated in separate curve analysis statements, with the curve analysis for reference curve before that of the experimental curve. For each curve, Parallelism Analysis is checked, and the curves identified as a reference (A) or as a sample curve with the reference curve being identified (B).

Results and discussion

Comparison of compounds to known hERG binding molecules can provide insight into whether prospective drugs will have this side effect. As demonstrated in Figure 3, a dose comparison of E_{410} with astemizole, a known hERG binding molecule, has a 10 fold greater EC_{50} . The relative potency of E_{410} was calculated to be 0.0683 of astemizole. While this indicates that this compound is less likely to bind than astemizole, the fact that it displaces the tracer indicates that it is a hERG-binding molecule.

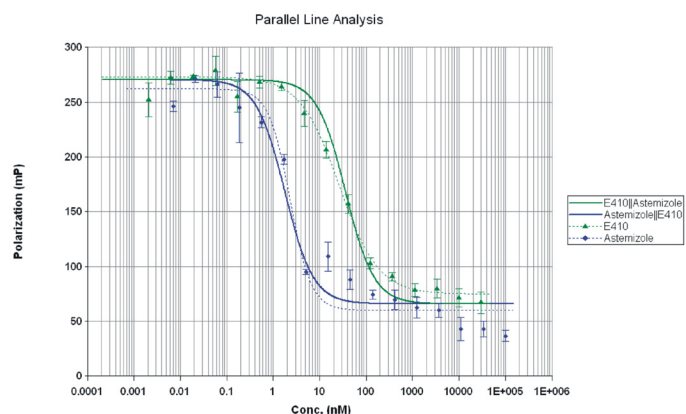


Figure 3. Parallel line analysis of two predictor fluorescence polarization curves.

With parallelism analysis, curve parameters for both individual curves are provided, as are parameters for the constrained curves. While the constrained curves have the same values for all parameters, the experimental curve has an extra parameter for relative potency (REP) that shifts the curve to the right or the left. As demonstrated in Figure 4, a potency value of less than 1 indicates that higher concentrations of the experimental agent are required to elicit the same response.

Curve Name	Curve Formula	A	B	C	D	REP	R ²	Fit Chi ² Prob	Para Chi ² Prob
E410 Astemizole	$Y = (A \cdot D) / (1 + (X/C)^B) + D$	270	1.42	1.8	65.8	0.0494	0.98	1.18E-026	1.13E-017
Astemizole E410	$Y = (A \cdot D) / (1 + (X/C)^B) + D$	270	1.42	1.8	65.8		0.958	1.18E-026	
E410	$Y = (A \cdot D) / (1 + (X/C)^B) + D$	273	1.03	26.4	75		0.992	0.822	
Astemizole	$Y = (A \cdot D) / (1 + (X/C)^B) + D$	262	1.88	2.3	60		0.959	2.68E-016	

Figure 4. Parallelism curve parameters. Curve parameters used to generate the constrained and unconstrained curves depicted in Figure 3.

Conclusion

The use of constrained curves makes comparing the potency of experimental compounds to reference compounds much easier. Care must be taken to ensure that the constrained curves adequately describe the curves in question. Using the R², Pearson χ^2 (when data weighting is used), and F-probability metrics, one can make a determination as to the reliability of the constrained curves. The Fisher-Snedecor F-test, or F-probability, is reported as a number from 0 to 1 and represents the probabilities that the sample and reference curves are parallel. In other words, the closer to 1.0 the value, the more likely the two curves are parallel. When data weighting has been used, specifically when 1/Std Y² or 1/s² weighting has been used, the Pearson χ^2 (Chi²) test can be used. The parallelism χ^2 probability is also reported as a number between 0 and 1, which represents the likelihood that the sample and reference curves are parallel. As with the F-probability, the closer the value is to 1.0, the greater the likelihood that the lines are indeed parallel.

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