The Use of Isothermal Titration Calorimetry in Antibody Quality Control and Characterization

MicroCal

ITC Application Note

Introduction

Antibody and antibody products play an important role in the development of many commercial products including biopharmaceuticals, diagnostic kits and chromatographic media. The quality control and characterization of these materials are of critical importance to the biopharmaceutical and diagnostic industries. Commonly employed methods of analyzing antibody product quality, such as electrophoresis or chromatography, characterize the molecular structure without providing information about binding activity. This is especially true in the evaluation of lot-to-lot variation of antibody function. Isothermal Titration Calorimetry (ITC) affords a much simpler and more accurate method for the quality control and characterization of antibodies and antibody products. ITC can be used to rapidly and effectively measure a wide range of physical characteristics, such as antibody affinity (K_a), the heat of antigen binding (ΔH), and the apparent number of active binding sites (n) on an antibody or antigen. Changes in structure (fragmentation, partial denaturation, and modification by coupling agents) can be correlated to changes in binding affinity. In addition, heats of binding can be used as predictors of the antibody-antigen reaction both *in vitro* and *in vivo*.

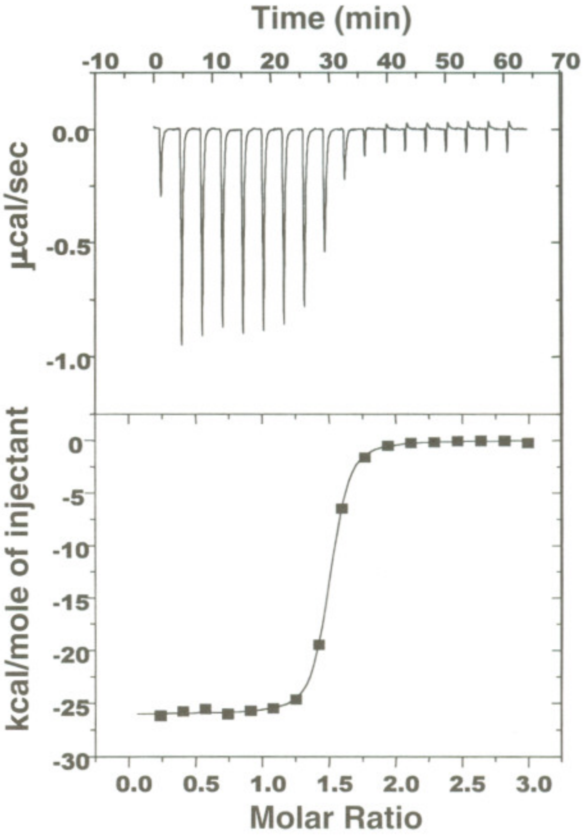

Figure 1. Representative ITC plot of an antibody experiment.

The top panel shows data obtained from individual injections. The bottom panel shows the areas under the peaks in the top panel, plotted as a function of a ratio of the moles of antigen added to moles of antibody in the cell.

In ITC, a solution of a ligand (e.g. a receptor, antibody, etc) is titrated against a solution of a binding partner at constant temperature. The heat released upon their interaction (ΔH) is monitored over time. As successive amounts of the ligand are titrated into the cell, the quantity of heat absorbed or released is in direct proportion to the amount of binding occurring. As the system reaches saturation, the signal diminishes until only heats of dilution are observed. A binding curve is then obtained from a plot of the heats from each injection against the ratio of ligand and binding partner in the cell (Figure 1). The MicroCal ITC system makes the characterization of binding simple with its automated robotic sample delivery system and computerized data collection and analysis. Individual experiments with our ITC instruments typically take less than an hour to perform. In this application note, ITC is used to measure the activity and affinity of a set of anti-quinidine and anti-theophyllin antibodies to their respective antigens. ITC is also used to demonstrate the deleterious effects which covalent immobilization has on anti-quinidine antibody activity. The high precision and reproducibility of the ITC method is demonstrated by replicate binding studies of the anti-theophyllin antibody to its antigen.

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Determination of Active Antibody Concentration

Theophyllin is a low molecular weight therapeutic agent used as a bronchodialator in the treatment of asthma (Figure 2). Other important medical applications include its use as a diuretic or as a cardiac stimulant. Antibodies against theophyllin are commonly used in diagnostic kits.

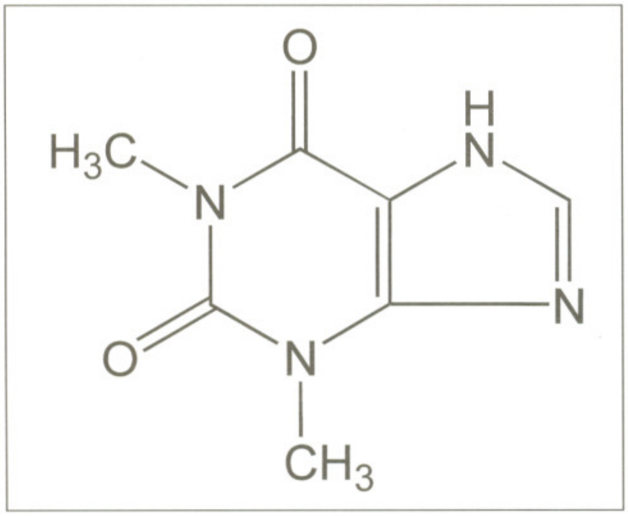

Figure 2. Structure of theophyllin (mol. wt. 180.17)

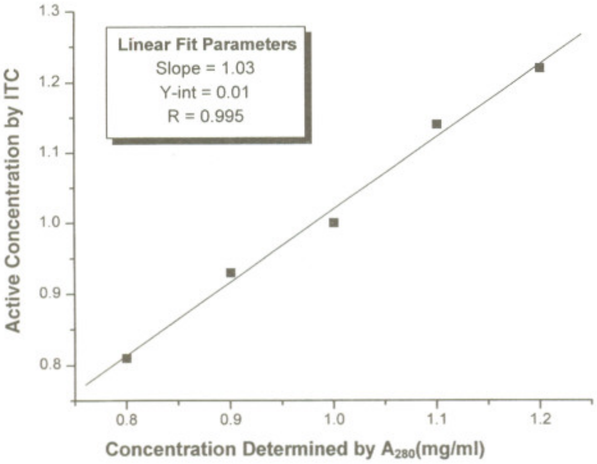

Figure 3. Comparsion of calculated versus expermentally determined antibody concentrations.

Studies were performed at 30°C. The volume of the sample cell was 1.3 mL. Five anti-theophyllin IgG antibody preparations with concentrations ranging from 0.8 mg/ml to 1.2 mg/ml were prepared by serial dilution. The concentration of the anti-theophyllin antibody stock solution was determined by absorbance at 280nm. For all experiments the concentration of theophyllin in the syringe was 160 μM. Each ITC experiment was performed with eighteen, 10μL injections and a 3.5-minute spacing between injections. A linear least-squares fit of the data is shown.

For the correct preparation of these kits, it is vital to accurately determine the activity of the antibodies utilized. We demonstrate here that ITC can be used to precisely determine the amount of *active antibody* in solution over a range of concentrations. The binding of theophyllin to five serially-made concentrations of an anti-theophyllin IgG was determined using ITC. In Figure 3, values of active concentration are plotted against total protein concentration, determined from absorbance spectroscopy at 280 nm.

A highly correlated linear relationship is observed between these two sets of measurements. The activity of the antibody in the solution can be determined by a ratio of the active concentration divided by the total protein concentration. For the data shown in Figure 3, the ratio is very close to 100%, suggesting a very pure antibody preparation.

Quality Control of Antibody Activity

In the biopharmaceutical and diagnostics industries, it is often vital to measure the quality and activity of different antibody lots. In this section, we demonstrate how the lot-

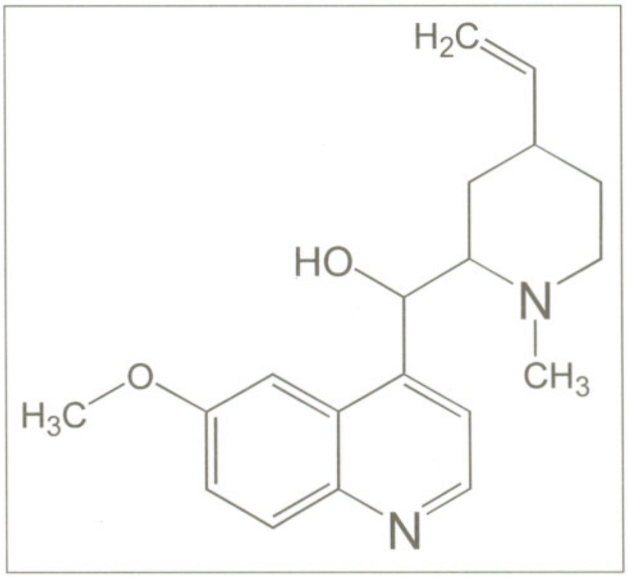

Figure 4. Structure of quinidine (mol wt. 324.41)

to-lot variability of antibodies can be determined easily and quickly using ITC, thereby discriminating between "good" and "bad" lots of an anti-quinidine antibody. Quinidine, the **R**-isomer of quinine (Figure 4), is a compound primarily used in the treatment of cardiac arrhythmia.Two different lots of an anti-quinidine antibody, provided by a leading manufacturer of diagnostic products, were evaluated by ITC in a blind study (Table 1). Titration studies against samples of these lots were performed under identical conditions and concentrations. As shown in Table 1, the binding activity of the bad lot is less than 15% of the good lot. These results were consistent with subsequent affinity chromatography studies, which showed the bad lot to exhibit substantially worse binding behavior than that of the good lot.

Antibody lot	Fraction Activity	K_a (x 10^8 M^{-1})	ΔH (Kcal/mol)
good	90%	3.3	-28
bad	12%	3.3	-28
immobilized	36%	0.16	-24

Table 1. Binding studies on anti-quinidine antibodies
For the good and bad lots, ITC measurements were performed using 1.3 ml of solution which was 6μM in antibody concentration, as determined by absorbance of the solutions at 280 nm. Concentration of the immobilized antibody was determined as the amount of antibody removed from the solution phase subsequent to attachment to the particles, also by measurements of absorbance at 280 nm. For all studies, successive 10μl injections of a quinidine solution (132 μM for the good and bad lots, 66 μM for the immobilized lot) were made at 4 min. intervals. The resulting data sets were analyzed in Origin in order to determine the binding constant K_a, heat of binding ΔH, and Fraction Activity.

Effect of Immobilization on Antibody Activity and Affinity

Antibodies against quinidine were covalently attached to iron beads. Beads with attached antibodies are typically stirred into a vessel containing heterogeneous solutions of antigenic materials. After the antigenic materials in solution bind to the antibodies on the beads, a magnet stationed near the vessel is used to separate and subsequently purify the bead-bound antigen. Sometimes, however, immobilization can affect antibody activity and affinity. When this occurs it is usually necessary to add increased amounts of bead-bound antibody. ITC is a convenient and reliable method to determine the amount of additional material necessary to overcome diminished activity. The immobilized lot which we studied was composed of good antibodies covalently immobilized to ferric oxide beads. As shown in Table 1, the apparent activity of the immobilized lot is approximately 40% of the good lot before immobilization. In addition, material immobilized to iron beads exhibits a 20-fold decrease in the binding constant (K_a) relative to that from the good antibody lot prior to immobilization. This suggests that the immobilization conditions used severely compromise anti-quinidine antibody binding affinity, probably due to the antibodies being bound in a manner which prevents optimal antigen binding.

Precision of ITC Method

In order to investigate the user-dependent variation of the ITC method for antibody binding, eleven identical binding determinations between theophyllin and the anti-theophyllin antibody were performed. For these experiments, the same operator used the same antigen and

	Activity	ΔH (Kcal mol^{-1})	K_a (x 10^7 M^{-1})	ln K_a
Mean	71%	-26.1	9.86	18.40
Std. Dev.	0.5%	0.3	1.07	0.11
CV	0.7%	1.1%	11%	0.7%

Table 2. Statistical parameters obtained from replicate ITC experiments on antibody-antigen
Eleven identical experiments were performed using the same antibody and antigen solutions. Each experiment consisted of twenty 5.0 μl injections of theophyllin solution (160 μM) into the sample cell containing 1.33 ml of a solution containing anti-theophyllin antibody (3.6 μM). Each data set was analyzed in Origin to obtain best values of parameters including the binding constant K_a, the heat of binding ΔH, and the % activity. The % activity was calculated as the number of binding sites determined experimentally for each antiobody molecule in solution, divided by the known number of binding sites per antibody molecule which is 2.0. Using these results from eleven experiments, the mean value, standard deviation, and coefficient of variation (CV) were determined for each of the three parameters as well as for the logarithm of the binding constant.

antibody solutions over a period of two days. From the data shown in Table 2, the variation of the ITC method for measurements of antibody activity and heats of binding are about 1%. For measurements of the affinity constant Ka of antibody-antigen binding, the variation of the ITC method is closer to 10% which is extremely high precision for this difficult parameter. This demonstrates that ITC is an extremely accurate and reproducible tool for the characterization of antibody activity and affinity.

Conclusions

Typically, the total protein content in a particular antibody lot is assayed spectrophotometrically and the activity is often measured using imprecise methods like agar diffusion. ITC can accurately and quickly determine active antibody content and binding constants. This information is helpful in determining antibody lot-to-lot variation, antibody characterization, and quality control parameters.

Binding parameters measured by ITC normally take between 30 minutes and one hour to determine. This is much less than methods like agar diffusion and equilibrium dialysis, which can take a day or more. In addition, user-dependent variation of ITC is extremely low, demonstrating that this method is highly reliable for the determination of many samples. Perhaps the most significant advantage of ITC is that it is a true in-solution method which requires no chemical modification of either binding component. Methods such as surface plasmon resonance and fluorometry require molecular modifications which might in turn produce large changes in the binding process itself. Such changes were seen to occur when the anti-quinidine antibodies were covalently attached to iron beads (Table 1).

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