

Summary

It is clear that calorimetric applications are able to encompass most of the demands of pharmaceutical drug discovery (see Figure 1). The improvements in sensitivity and instrument design have begun to allow significantly reduced sample size to be used. Furthermore, the move toward high throughput instruments is underway which will undoubtedly lead to calorimetry becoming the method of choice in the quest for new and improved pharmaceutical products.

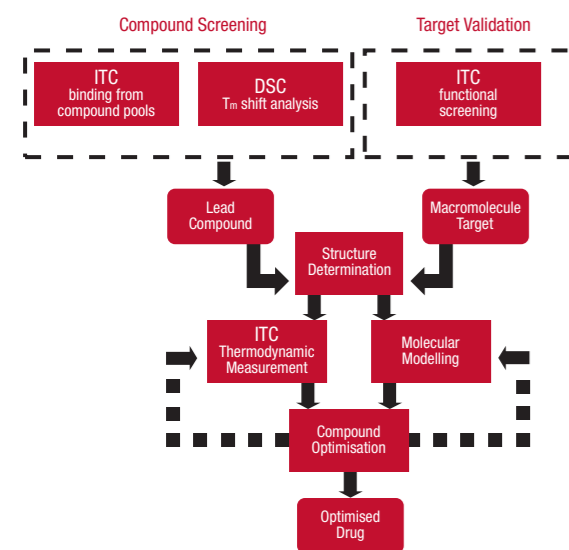
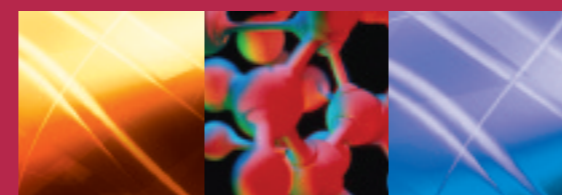


Figure 1. Calorimetry in the Drug Development Process

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www.microcalorimetry.com

MicroCal, LLC
22 Industrial Drive East
Northampton, MA 01060
United States of America
T +1 413-586-7720 / 1-800-633-3115
F +1 413-586-0149
E info@microcalorimetry.com

MicroCal, LLC Europe
2 Warren Yard, Warren Farm Office Village
Wolverton Mill, Milton Keynes MK12 5NW
United Kingdom
T +44 1908 576330
F +44 1908 576339
E info@microcal.eu.com



Calorimetry Takes the Heat Off Drug Discovery

Application Note

Written by:

Dr John E. Ladbury

**Department of Biochemistry and Molecular Biology,
University College London, Gower Street, London, WC1E 6BT, UK.**

Tel: +44 20 7679 7012 Fax: +44 20 7679 7193 Email: j.ladbury@biochem.ucl.ac.uk

Reprinted from European BioPharmaceutical Review Winter 2001 Edition Pages 70-72





There are two essential steps at the beginning of the process of bringing a drug compound to market; the identification of a lead compound, and the optimisation of this lead compound to hone the required characteristics, including its affinity, for its specific target. The modern pharmaceutical industry is involved in a continuous search for ways of streamlining these two steps to increase yields, decrease time and reduce costs. This has meant that there has been a pressure to develop the capabilities of instrumentation to assist in these processes. The recent revolution in the development of calorimetric instrumentation, and its applications, has led to it now taking a place in the frontline in the battle to develop pharmaceutical products.

Calorimetry In Drug Screening

The modern process of screening for candidate lead compounds involves rapid identification amongst a pool of molecules one or more that bind to a target molecule. To be able to do this efficiently a sensitive signal which highlights the presence of an interaction is required. Currently popular methods include monitoring interaction using fluorescent or radioactive labelling. An alternative approach is beginning to evolve whereby the heat of interaction is used as the probe for a compound binding to the target molecule. With the development of high sensitivity calorimeters these instruments are being adopted as a tool in drug screening.

Methodologies utilising both the isothermal titration calorimeter (ITC) and the differential scanning calorimeter (DSC) instrumentation are currently being developed.

I) Applications of ITC for Drug Screening

The ITC instrument provides a highly sensitive probe of heat of interaction at a given temperature. Injection of an interacting drug into the target molecule (which is accommodated in the calorimeter cell) will result in an endothermic, or exothermic, enthalpic event which will be observed as a heat pulse. Thus, if a pool of molecules is injected the presence of any interacting molecules, or 'hits', can rapidly be observed by the change in enthalpy. As with other screening procedures, by using different matrices of molecules from compound libraries it is possible to rapidly identify the hits. This method of identifying binding

compounds is superior to many other techniques since it precludes the necessity for chemical modification of the target or drug compound (e.g. attaching fluorescent tags or radio-active labelling). Furthermore, since every biomolecular interaction is accompanied by a change in enthalpy it should be an ubiquitous probe. Current limitations to the widespread adoption of this methodology are based on the perceived material requirements and the lack of instrumentation to allow high throughput of molecules. These problems are being addressed currently by instrument manufacturers. With regard to the amounts of material required, the screening process only requires detection of interaction rather than the determination of the absolute affinities (or full thermodynamic characterisation). As a result, it is only necessary to observe a single heat pulse to identify a binding event. Since heat can be measured to such high sensitivity, typically concentrations down to the picomolar range are sufficient to detect binding.

Development of this type of approach has stimulated a great deal of interest from pharmaceutical scientists including academic/industrial partnerships in my own laboratory. An example of the potential use of this methodology in drug discovery, The Althexis Company, Inc. (Waltham, MA, USA) are currently successfully using this calorimetric approach to screen libraries of compounds to identify enzyme activity for target validation.

II) Applications of DSC for Drug Screening

Use of DSC in the screening process has also begun to raise considerable interest. DSC can measure the change in heat associated with a thermal transition as a prescribed temperature range is scanned. So for example, the change in enthalpy and the T_m (the temperature at which the equilibrium constant, K , for the unfolding/folding change for a two state transition is equal to 1) for the unfolding of a protein can be measured accurately. Determination of T_m values for target macromolecules provides the basis for compound screening. When a protein unfolds in the presence of a bound ligand the value of T_m will increase in comparison to unfolding without ligand, due to the effect of coupled equilibria. Since in modern DSC instruments the T_m can be measured to high accuracy (> 0.1 C), this provides a highly sensitive probe of binding. In a typical experiment the melting profile of a target macromolecule (protein or DNA) can be obtained using DSC, and then rescanned in the presence of a pool of candidate, small molecular weight ligands. A shift in the T_m value of the macromolecule as shown in the DSC scan will

provide evidence for the binding of one of the pool. Again using a matrix pooling approach the binding compound can be identified and hence, a lead compound discovered.

The success of this approach was demonstrated in a paper given at the Biocalorimetry 2001 Conference in Philadelphia by Professor C. B. Chaires (University of Mississippi, Jackson, MS, USA). Using a large 2.5 kilobase DNA sequence containing the binding sequences for preferred drug binding domains, the change in T_m for a given peak in DSC scan could be identified and hence the binding preferred binding site of a drug compound from a large pool of compounds identified. This method of "boot-printing" could be applied to screening large numbers of potential ligands to interact with oligonucleotide targets.

Again the issues of the concentrations of protein required and the lack of high throughput capability have been cited as possible hindrances of the widespread adoption of this methodology in pharmaceutical discovery programmes. The modern generation of DSC instrumentation is highly sensitive (capable of measuring down in the nanocalorie range) which reduces the amounts of material required. Furthermore, since a screening scan only requires that a shift in T_m be observed (rather than be used to determine the thermodynamic parameters of unfolding which the technique has historically been used for) experiments can be done at concentrations comparable with other screening methodologies. With regard to the high throughput capacity, new generation calorimeter design is likely to take this into consideration.

Calorimetry In Structure-Based Drug Design

Structure-based drug design is a general term applied to the process whereby a high-resolution structure of the target protein (or DNA) is used to provide information on how a potential ligand might interact with a surface. In its simplest form this can be done by taking a compound and visually inspecting ways in which it will fit into a preordained binding site on the target molecule. By assessing which interactions in the binding interface are likely to be favourable and which are unfavourable, suggestions for compound improvement can be rationally made. Quantification of these favourable and unfavourable interactions requires knowledge of the thermodynamics of the interaction. The rational design process has become increasingly sophisticated over the last ten years, such that now complex docking algorithms and molecular dynamics simulations can be used to predict binding energies

for positioning drug compounds in target binding site. In an ideal scenario these data are fed to medicinal chemists who can modify the compounds to optimise the interactions in the interface. The capability to provide useful information is seriously hampered by a lack of experimental thermodynamic data with which to verify these sophisticated programs, and to enable checking of the effects of molecular variation in the drug discovery process. Furthermore, this form of drug discovery is based only on the prediction of the change in free energy (ΔG) of an interaction. This is generally assimilated by the addition of all of the non-covalent bond terms in the interaction derived from the theoretical enthalpy changes (ΔH) based on bond distance. The entropic terms, including those derived from the effect of solvent interactions, are usually ignored in such programs.

Using ITC highly accurate determination of the thermodynamic parameters of a biomolecular interaction can be obtained. ITC provides a method for the measurement of the ΔH of an interaction at constant temperature and pressure (for reviews of the method see for example¹⁻⁷). Since calorimetric methods can measure directly the ΔH of an interaction at a given temperature directly they, avoid the indirect calculation of this term necessary in other methods (e.g. spectroscopic techniques), which is inherently less accurate. In ITC the enthalpy of the interaction is measured as one component of an interaction is titrated into the other as the binding sites are saturated. The heat change of interaction (exothermic or endothermic), therefore, is a probe of the amount of bound ligand at each injection. Therefore, from the ratio of bound and free interactants the binding constant can be determined⁸. Having established these two parameters at a given experimental temperature (T) the free energy can be determined using equation 1:

$$(1) \Delta G_{obs} = -RT \ln K_{B,obs}$$

Furthermore, knowing the (G_{obs} and the (H_{obs} the change in entropy, (S_{obs}), can be calculated, from equation 2:

$$(2) \Delta S_{obs} = (\Delta H_{obs} - \Delta G_{obs})/T$$

Thus, in one experiment a full thermodynamic characterisation of the interaction at a given temperature can be obtained. In the ITC experiment the heat of interaction measured includes contributions from all the equilibria that occur on the interacting components going from the free to bound state. Thus, the thermodynamic terms are considered as 'observed' to signify that they are derived from experiment and the subscript 'obs' is inserted. If the enthalpy is measured at a range of temperatures

then the change in constant pressure heat capacity ($\Delta C_{p,obs}$) can be determined based on the following equation:

$$(3) \Delta C_{p,obs} = (\Delta H_{T1,obs} - \Delta H_{T2,obs})/(T2 - T1)$$

where T1 and T2 are two different experimental temperatures. The change in heat capacity is affected by several important contributions which characterise binding. Correlation of ΔC_p with burial of surface area in the binding site has been used as a useful tool in drug discovery^{9,10}

The value of this experimental data in its application to the drug design process is beginning to be revealed. ITC has been applied in the challenging area of optimising inhibitors to the Src SH2 domain¹¹ by the group at Glaxo Wellcome Inc. (latterly GSK Inc.) NC, USA . Thermodynamic data from ITC was used by Walter Ward and colleagues at AstraZeneca to aid in their understanding of binding to their gyrase target protein¹². Currently, several pharmaceutical companies are integrating thermodynamic experimental data from calorimetry into the drug design process. As Jim Thomson of Agouron/Pfizer, La Jolla, CA, USA is keen to point out, "ITC is now a major player in our arsenal of techniques to give feedback to the drug design process".

As a further advertisement as to the variety of applications currently being developed by the pharmaceutical industry ITC is finding a niche as a tool in kinetic studies with potential to assess inhibition characteristics. As Matt Todd of 3-D Pharmaceuticals Inc. Exton, PA, USA (in collaboration with Prof. E. Freire of Johns Hopkins University, Baltimore, MD, USA), clearly demonstrated at the Biocalorimetry 2001 Conference, there is a great deal of scope for the capability of ITC instrumentation to accurately measure the heat of turnover of a substrate by an enzyme and therefore provide a direct probe of the rate of reaction.