Stability Assessment of Pharmaceuticals and Biopharmaceuticals by Isothermal Calorimetry

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Abstract: The assessment of stability (of actives, excipients and/or formulated products) is an important, and often time-consuming, part of pharmaceutical product development. Conventionally, HPLC is used to quantify the concentrations of a parent compound and any degradation products as a function of storage time. HPLC, however, is relatively insensitive to small changes in concentration and it is often the case that stability assays are conducted under stress conditions, in order to accelerate any degradation processes. The Arrhenius relationship is then employed to give an initial prediction of stability under storage conditions while long-term studies, under storage conditions, are conducted to confirm these predictions. The properties of isothermal calorimetry, such as its intrinsic sensitivity to small changes in heat and invariance to the physical form of a sample, make it ideally suited for stability assessment because it obviates the need for an Arrhenius analysis. In addition, the ability to conduct titration or gas perfusion experiments vastly increases its range of applications. Recent advances in instrumental design and data analysis have made it easier to analyse data quantitatively for complex systems. It is the purpose of this review to highlight some of these developments, discuss them in the context of pharmaceutical and biopharmaceutical examples and explore some of the future challenges and applications of the technique.

Key Words: Isothermal calorimetry, drug stability, excipient compatibility, amorphous content, stability assessment, pharmaceuticals, biopharmaceuticals.

1. INTRODUCTION

The physical characterisation of a new drug substance, or active pharmaceutical ingredient (API), is an important step in the formulation process of a new pharmaceutical product and usually occurs after assays have shown the API to be efficacious and essentially (all drugs have side-effects) non-toxic. Such characterisation may include the determination of solubility and p*K* (if the drug is an acid or base), the detection and quantification of different polymorphic forms of the drug and the kinetics of any degradation reactions that may be occurring. Once characterized, and if the drug has suitable properties, the next challenge is to formulate it into a medicine, an often time-consuming process involving many steps. One of those steps is an assessment of the stability of the product, which is required to ensure that the medicine performs within pharmacopoeial specifications during its stated shelf-life. There are many potential reasons why the performance of a medicine may lessen upon storage; the drug may degrade, either independently or by interaction with one or more excipients, to an inactive (or, worse, toxic) derivative, the polymorphic form of the drug may change, altering its degradation kinetics and dissolution rate, or the excipients may degrade or interact, altering the mechanical action of the product.

HPLC is the principal technique used in the pharmaceutical industry to assess drug stability by quantifying the concentrations of the parent compound and any degradation products as a function of storage time. However, the use of HPLC is limited by two important factors; its relative insensitivity to small changes in concentration and the requirement that samples are dissolved in a suitable solvent prior to analysis. The former is a problem for all samples that exhibit slow degradation rates while the latter can affect samples not formulated in solution, as accelerated decomposition rates are often observed when compounds are solvated.

Because of the poor sensitivity of HPLC, it is often the case that stability assays are conducted under some condition of elevated stress, the applied stress usually being an increase in temperature and/or relative humidity. The resulting increase in degradation rate allows a rate constant (k) to be determined at each temperature, although study periods may still extend for weeks or months. The data are then plotted in accordance with the Arrhenius relationship ($\ln k$ versus $1/T$) and the predicted rate constant under storage conditions is obtained. Necessarily, it is assumed that the analysis results in a linear relationship and that any reaction processes occurring under stress conditions are the same as those that would occur under storage conditions. There are many reasons why this may not be so; the reaction may not follow first-order kinetics, different reaction pathways may predominate at higher temperatures, the sample may change its crystal state or go through a glass transition or water may be involved in degradation. If there is any doubt about the extrapolation, then long-term storage studies (holding the sample directly under expected storage conditions) are conducted to confirm the findings.

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HPLC analysis of an API cannot, of course, detect if a solid drug has changed polymorph, because dissolution of the sample before analysis removes any solid-state history, nor will it be of use if it is a change in the properties of an excipient, or an interaction between excipients, that causes the product to fail to meet its specification.

Isothermal calorimetry (the measurement of heat-flow as a function of time) offers a better approach to stability assessment because it is sensitive enough to allow the analysis of samples non-destructively (that is, it does not cause any extra degradation than that which would have occurred upon storage), directly under storage conditions. Moreover, because both physical and chemical processes occur with a change in heat content, the technique is not limited in its detection ability to chemical degradation, in the way HPLC is. This unique combination of qualities makes isothermal calorimetry ideal for pharmaceutical stability assessment.

Careful experimental design allows the investigation of virtually any system and recent advances in data analysis and interpretation methodologies have resulted in the increasing application of the technique to stability testing of pharmaceuticals. Indeed, careful data analysis can result in a description of the reaction process in terms of both thermodynamics and kinetics, the only technique for which such a complete analysis is possible. It is the purpose of this review to highlight some of these developments and applications, discuss them in the context of pharmaceutical formulation and explore some of the future challenges and applications of the technique.

2. THE PRINCIPLES OF ISOTHERMAL CALORIMETRY

In isothermal calorimetry (IC), the heat-flow to or from a sample (power, φ, in μW) is measured as a function of time (s). Integration of the power-time data gives the change in heat content (enthalpy, ΔH, in μJ).

Since heat is universal, virtually any sample can be studied using the technique, as long as a representative sample can fit into an ampoule, and the heat-flows of all the processes, chemical or physical, occurring within the sample are recorded. This allows the investigation of complex systems that would normally fall outside the scope of traditional analytical techniques, but often results in complex data that are difficult to interpret. It also means that sample preparation is paramount; careless sample preparation may result in erroneous heat-flows which will subsequently prevent accurate data interpretation.

In IC any heat produced or absorbed by a sample is, ideally, completely exchanged with a surrounding heat-sink, maintaining the sample at a constant temperature. Usually, a reference ampoule is loaded with an inert material, of similar heat capacity and in a similar quantity to the sample, and data are obtained as a differential response between sample and reference. Consequently most of the noise arising from temperature fluctuations is removed when the reference data are subtracted. A modern, heat-conduction, isothermal calorimeter (such as a TAM III housing a 3201 nanocalorimeter, Thermometric AB, Järfälla, Sweden) is capable of maintaining a baseline stability of $< \pm 20$ nW over 24 h with a temperature accuracy of ± 0.0001 ^{o}C.

2.1. Calibration

An important issue, especially for the pharmaceutical industry, is instrument validation. Calorimeters are usually calibrated periodically using an electrical substitution method, whereby a resistance heater near the sample ampoule supplies a known amount of heat for a defined period of time. The recorded heat output measured by the instrument is adjusted to match the programmed heat input. Clearly the heat-conduction pathways in such a calibration do not match accurately those of a real sample unlike, for instance, differential scanning calorimeters that use certified chemical standards, such as indium. This has led to a recent IUPAC report of standards for isothermal microcalorimetry [1]. Chemical test reactions which, in principle, offer a more realistic test of the operating performance of microcalorimeters include the imidazole catalysed hydrolysis of triacetin for isothermal instruments [2-6], the base catalysed hydrolysis of methyl paraben for flow microcalorimeters [7] and the Ba^{2+}/18-crown-6 interaction for titration instruments [8].

3. STABILITY ASSESSMENT

The primary aim of stability assessment is to ascertain whether a compound, either alone or in combination with other actives or excipients, will degrade significantly over a defined period of time under specified environmental conditions. If no degradation is observed then the compound is assumed to be stable and no further assessment is necessary. If degradation is observed then either the system is abandoned or further experimentation is required so as to identify the cause (which may not necessarily arise from a chemical change in the sample; physical changes are likely in amorphous or polymorphic drugs or in heterogeneous drug delivery systems).

3.1. Primary Screening

It has been shown that, for a reaction following first-order kinetics and occurring with a reasonable ΔH of -50 kJ mol^{-1}, it is possible to distinguish between rate constants of 1 x 10^{-11} and 2 x10^{-11} s^{-1} by analysis of 50 h of power-time data recorded at 25 ^{o}C using an isothermal calorimeter [9]. A reaction progressing with a rate constant of 1 x 10^{-11} s^{-1} has a half-life of approximately 2,200 years; given that degradation of an API or excipient will lead to a loss of potency of a product, and that the lowest acceptable level of potency is usually 90% of the label claim [10], the potential of calorimetry to assess drug degradation is immediately apparent. Moreover, this result is obtainable by recording data over 2 days under storage conditions.

Thus, an initial screen of a sample, conducted by loading a sample of the pure material into an ampoule and measuring the heat-flow as a function of time, should detect rapidly any degradation processes occurring and the absence of any heat responses gives confidence that the system under investigation is stable (although it should be noted that exothermic and endothermic heat-flows occurring simultaneously will reduce the observed net power signal). Such an approach

was first described by Pikal [11], who showed there was a correlation between the exothermic heat output of some pharmaceutical systems and their known degradation rates (previously determined using other analytical methods). The data also showed that degradation rates of the order of 2% per year were easily quantified.

A similar approach can be adopted for testing binary mixtures of API and excipient [12-13]; the thermal behaviour, under a controlled relative humidity (for example, 75%), of the active alone and the excipient alone are recorded and compared with that determined for a binary drug-excipient mixture. Any unexpected heat-flows recorded in the drug-excipient mixture indicate a possible interaction. Note here that these initial screens are usually designed to maximise the chance of seeing an interaction, if there is one, rather than match the conditions to be found in the intended formulation; thus, binary samples are usually mixed in a 1:1 by mass ratio, ensuring the two materials have equivalent particle sizes, and a high RH is used. Further screens under more representative conditions can then be conducted if required.

A further adaptation of this approach is to use water slurries instead of humidified samples, [14-15]. Schmitt *et al.* [14] developed a procedure that allows rapid assessment of API-excipient compatibility by studying two developmental drugs formulated with excipients that could undergo a Maillard reaction. Their recommended methodology is to add water (20% w/w) to a binary mixture of API and excipient (100 mg of each) and monitor the power-time signal at 50 ^{o}C for 3 days. They noted that comparison of the calorimetric results with actual formulation stability showed it was possible to predict relative stability within functional classes, but advised caution because the apparent reaction enthalpies varied three-fold among excipients within the same functional class.

It should be mentioned that differential scanning calorimetry (DSC) has also been used as a tool to conduct primary compatibility screens [For example, 16-21]. As for IC, the thermal responses for the API and excipient alone are compared with those recorded for the API blended with a range of excipients. Any changes in the expected peaks, or the appearance of new thermal events, indicates a likely incompatibility. For instance, ibuproxam has been shown to be compatible with corn starch, avicel and sodium carboxymethylcellulose but incompatible with PEG 4000, stearic acid and magnesium stearate using DSC data [22]. A similar approach, using DSC in a step-isothermal mode, has been used to study the known incompatibility between aspirin and magnesium stearate [23]. The major benefit of using DSC for primary screens is one of time; an initial judgment on likely incompatibilities can be made in a few minutes. However, the same caveat applies to the interpretation of DSC stability data as for all elevated temperature stability assessments; it must be assumed that the reaction mechanism doesn't change as a function of temperature.

The approach adopted in the studies discussed above (both IC and DSC) does not allow the quantification of the amount of degradation (unless complete degradation occurs within the time frame of the experiment) nor, without further analysis, does it indicate the exact nature of any degradation processes. It does, however, allow an initial judgment to be made on the likely stability of a compound or mixture, giving the formulator valuable insight into the stability of the API to be formulated or which excipients are likely to result in a stable product. As such, the use of IC during initial product formulation offers the potential greatly to reduce the number of potential formulations undergoing stability assays, although IC data alone are not currently accepted by regulatory bodies to prove product stability. IC data can, however, aid in the continuing development and product line extension of existing products, whose stability has been assessed and accepted using classical methods. In this case, the thermal response from the API with new excipients and/or packaging materials can be recorded; the lack of a detectable heat signal provides good evidence that no new incompatibility reactions will be introduced into the new product and that the existing stability data are still applicable.

The lack of quantitative studies is a direct result of the difficulties that arise in the analysis of complex power-time signals: for instance, a low enthalpy, fast rate process may easily appear the same as a high enthalpy, slow rate process [24], or competing endothermic and exothermic processes may result in an apparently small thermal response [25]. These problems were noted in the study of API-excipient stability using water slurries by Schmitt *et al.* [14], and made direct stability assessment comparisons between samples difficult. While a slow rate process may have no consequence on product stability a fast rate process may be of some considerable significance and it is clear that an analysis that results in the derivation of a rate constant, reaction enthalpy or both is necessary for true comparisons to be drawn. Indeed, it has been stated that although IC offers considerable benefits in determining product stability, ultimately it will never replace the need for chemical analysis [26]; this area forms a considerable challenge that must be overcome if IC is to become more widely used for pharmaceutical stability assessment.

3.2. Stability Assessment of Individual Compounds

If the primary screen conducted in the calorimeter indicates some incompatibility or instability in the sample, and there is a need to understand the process on a molecular level or define its reaction kinetics and/or thermodynamics, then the traditional approach is to conduct further analyses with complementary analytical techniques. However, it is usually the case that the power-time data so obtained already contain sufficient information that, if a suitable analysis can be undertaken, the process(es) occurring can be quantified with no further experimentation.

If the degradation process under investigation is known, or suspected, to follow first-order kinetics, a simple approach to data analysis is to plot ln(power) versus time [27-31]. The initial gradient of the resulting linear plot gives the first-order rate constant directly.

For instance, the degradation rate of meclofenoxate hydrochloride (MF), which hydrolyses in aqueous solution, has been determined using IC [29]. By plotting ln(power) versus time, the degradation rate constants for MF at pH 6.4 and 2.9 were determined to equal 1.14 x 10^{-4} s^{-1} and 9.7 x

10^{-7} s^{-1} respectively. Comparison of these data with rate constant values determined using HPLC (1.29×10^{-4} s^{-1} and 9.0×10^{-7} s^{-1}) revealed the utility of the calorimetric technique. A similar approach has been used to determine the rate constants for ampicillin degradation in aqueous buffers from pH 2 to 8 [30] and a number of cephalosporins [31].

Koenigbauer *et al.* [32] determined the activation energies for the degradation of several drugs, including phenytoin, triamterene, digoxin, tetracycline, theophylline and diltiazem, using the initial heat output rates, measured using IC at several elevated temperatures. The results were compared with HPLC data recorded at a single temperature and it was shown that the IC data were more precise. Similarly, Hansen *et al.* [33] showed that the shelf-life of a product, degrading via an autocatalytic reaction, was inversely proportional to the rate of heat production during the induction period, using lovastatin as an example.

Since most microcalorimeters employ closed ampoules, it is possible to control the local atmosphere in the sample cell and hence to study oxidation reactions. A simple methodology to determine the presence of oxidative degradation is to run samples in closed ampoules under either air or nitrogen; differing power-time profiles indicate that oxidation is occurring. For example, varying the oxygen content in an ampoule containing an aqueous solution of ascorbic acid varies the power-time signal obtained [34-35], indicating the presence of an oxidative process. Ascorbic acid oxidises reversibly in aqueous solution to form dehydroascorbic acid which is subsequently irreversibly hydrolysed to give diketogluonic acid. Angberg *et al.* [34] noted that the heat-flows measured for solutions of ascorbic acid in pH 4.9 and pH 3.9 buffers were greater for those samples that were prepared with an air space in the ampoule compared with those that were not. Furthermore, if the solution was purged with nitrogen prior to loading, the heat-flow dropped nearly to zero. Willson *et al.* [35] noted, by observing a linear ln(power) vs. time plot, that the oxidation was first-order with respect to oxygen concentration. Both studies suggested that the oxygen in the ampoule was exhausted after 3-4 h by virtue of the fact that the measured heat-flow after this time reduced to zero.

The rate of ascorbic acid degradation is affected by a number of other factors including pH, ascorbic acid concentration, the presence of metal ions and the presence of antioxidants. Angberg *et al.* [34] observed that the measured heat-flow increased with increasing ascorbic acid concentration up to a certain concentration whereupon further increases did not increase the power response. It was presumed the reaction became limited by the oxygen concentration at higher acid concentrations. Willson *et al.* [35] calculated the rate constants for the oxidation at varying ascorbic acid concentrations and found they were identical, concluding that the oxidation rate is independent of acid concentration. The presence of metal ions (copper or iron, for example) is known to affect the oxidation rate of compounds in solution and is difficult to control, because only trace amounts are required to catalyse the reaction. Both authors conducted experiments in the presence of EDTA, a metal chelating ligand, and observed that the measured heat-flows fell substantially compared with samples run in the absence of the metal binder.

Similarly, the solid-state degradation pathways of 13-*cis*-retinoic acid and all-*trans*-retinoic acid have been shown to differ in the presence and absence of oxygen [36]. Under an air atmosphere, 13-*cis*-retinoic acid follows an autocatalytic reaction pathway, while all-*trans*-retinoic acid undergoes a zero-order degradation process. These processes were also followed by HPLC analysis which confirmed the reaction kinetics. Under a nitrogen atmosphere both samples showed a first-order power-time trace. HPLC analysis showed no evidence of drug degradation under these conditions, implying the calorimeter was recording a physical change in the sample. A similar approach has shown that solid-state lovastatin degrades in the presence of oxygen [28]. In this case, the degradation mechanisms were shown to change between 50 and 60 ^{o}C, an important observation in the context of elevated temperature studies, as discussed earlier.

For more complicated reaction processes, occurring in solution, it is possible to analyse calorimetric data using models based on classical reaction kinetics. The underlying principle in this case is that the heat released by the process under investigation at time t (q_t) equals the enthalpy change for that process (ΔH) multiplied by the number of moles reacted at time t (x) [37-38]. Substituting x with $q_t/\Delta H$ into an integrated rate expression and rearranging gives a kinetic expression that describes power-time data. For a first-order reaction, it can be shown [37-38] that;

$$\frac{dq}{dt} = \text{Power} = \Delta H.V.A_o.e^{-k.t} \qquad \text{Equation 1}$$

where V is the volume of the sample, A_o is the number of moles of reactant available for reaction and k is the first-order rate constant. This method of analysis was first applied to the acid hydrolysis of methyl acetate in hydrochloric acid [37]. The experimental data were fitted to a first-order kinetic expression using a least squares analysis, which returned values of $k = 0.116 \times 10^{-3}$ s^{-1} and $\Delta H = 1.98$ kJmol^{-1}.

It is also possible to use this approach to study the degradation of APIs in solution. For instance, aspirin degradation in aqueous solution follows pseudo first-order kinetics and can be successfully analysed using Equation 1. Fig. **1** shows the power-time traces obtained when aspirin degrades in 0.1M HCl solution at 25, 40 and 50 ^{o}C and the fit of each trace to Equation 1.

A similar approach has been used to analyse the solution phase degradation of potassium hydroxylamine trisulfonate in acid solution [39], which degrades to hydroxylamine via two intermediate species. Power-time data, obtained at 25, 30 and 35 ^{o}C, were fitted to a three-step consecutive model and returned values for the three rate constants and reaction enthalpies. The data were also fitted to a simpler two-step model. The simpler model described the initial data well, presumably because the third hydrolysis reaction did not occur significantly during this period, but could not fit the entire curve. An important result of this study is that the models possess sufficient sensitivity to determine mechanistic information. This is of some considerable importance for it offers the potential to extract mechanistic information

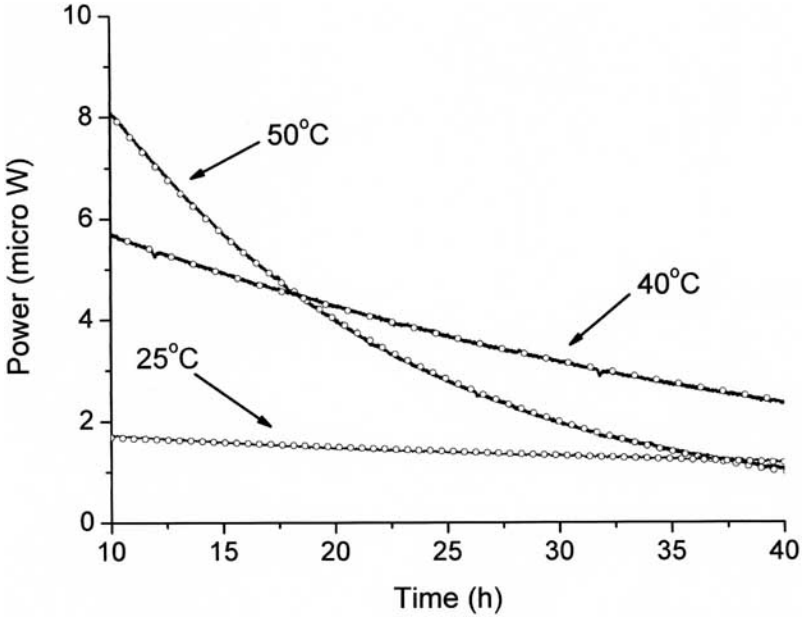

Fig. (1). Power-time data for the degradation of aspirin (0.01 M) in HCl aqueous solution (0.1 M) at 25, 40 & 50 ^{o}C (solid lines) and the fit lines (o) generated by application of Equation 1 [Skaria and Gaisford; unpublished results].

from a study of the power-time profile for an unknown reaction (by fitting data to a series of mechanistic models and noting which gives the best fit).

This methodology also allows the analysis of compounds degrading in parallel. Skaria *et al.* [40] have demonstrated that the rate constants of degradation of methyl, ethyl and propyl paraben are different when they are formulated in binary pairs as opposed to being in solution individually. This is an important observation, not least because the parabens are usually formulated in combination, but also because it demonstrated that the kinetic models described above are sensitive enough to detect subtle changes in degradation rates and pathways. In the context of determining the stability of complex, heterogeneous pharmaceuticals this approach may, ultimately, obviate the need for traditional chemical analyses, such as HPLC or GC.

A similar methodology can be used to investigate solid and semi-solid samples, although for these systems the major problem lies in defining the underlying kinetic expression for the process. Solution phase kinetics can be described in molar terms while solid phase kinetics are usually written in terms of the fraction of material reacted. The kinetics of semi-solid systems are complex and not easily defined.

One approach that has been used to analyse the degradation of solid state samples utilises the Ng equation, an equation said to describe all solid-state reaction processes [41]. Conversion of the Ng equation to a form that describes calorimetric data yields [9];

$$\frac{dq}{dt} = A_o.k.\Delta H.(\frac{q}{A_o.\Delta H})^m.(1-\frac{q}{A_o.\Delta H})^n \qquad \text{Equation 2}$$

where *m* and *n* are fitting constants, the values of which relate to the underlying reaction mechanism. Willson *et al.* [42] used Equation 2 to analyse the solid-state degradation of ascorbic acid and samples of ascorbic acid with small quantities of added water. Dry ascorbic acid degraded with a rate constant of 1.15 x 10^{-6} s^{-1} and an enthalpy of -199 kJ mol^{-1}. Addition of small aliquots of water (from 20 to 200 µl to 0.5 g of acid) did not significantly change the reaction parameters. At added water amounts of 500 µl and greater the reaction kinetics were best described by a solution phase model. Between 200 and 500 µl of added water the data were not described by either model, suggesting a complex, mixed phase system had been formed. Equation 2 has also been used to analyse a physical process, the isomerisation of nitritopentamminecobalt (III) chloride [9], giving a rate constant of 2.98 x 10^{-6} s^{-1} and an enthalpy change of -2.99 kJ mol^{-1}. Further theoretical aspects of the analysis of solid-state reactions using IC have been discussed by O'Neill *et al.* [43].

An inherent assumption made in analyses based on the approach discussed above is that *the entire* sample placed in the ampoule will react, *i.e.* that the reaction progresses to completion and no equilibrium state, involving both reactants and products, is reached. This assumption is necessitated by the use of an iterative procedure to return values for the unknown reaction variables and clearly restricts the applicability of the methodology.

Recently, a non-iterative method that allows the determination of reaction parameters from microcalorimetric data has been published [44-45]. In this case, the initial approach is to record power-time data for the system under investigation at different temperatures. The total heat output, *i.e.* that which would be recorded if the whole sample that *could* react did react (which is not necessarily the entire sample in the ampoule), for the reaction (*Q*), is calculated at each temperature. If the value of *Q* remains constant then no equilibrium state is involved, the reaction progresses to completion and ΔH can be determined directly. If the value

of Q varies, then an equilibrium state exists and the equilibrium constant must be determined. It can be shown [44] that Q_T, the heat output if the reaction went to completion, for a first-order A ⇔ B reaction is given by;

$$Q_T = (Q_2^2 Q_1 + Q_2^2 Q_3 - 2Q_1 Q_2 Q_3)/(Q_2^2 - Q_3 Q_1) \quad \text{Equation 3}$$

where Q_1, Q_2 and Q_3 are the total heat outputs at temperatures T_1, T_2 and T_3 respectively assuming that the same amount of sample (A_T) is used in each experiment. This expression, derived from the van't Hoff relationship, holds only if T_1, T_2 and T_3 are chosen such that the following equality holds;

$$T_1 T_2/(T_2 - T_1) = T_2 T_3/(T_3 - T_2) \quad \text{Equation 4}$$

A convenient set of sample temperatures that fulfils this requirement is T_1 = 293 K, T_2 = 298 K and T_3 = 308.5 K. From these data, the equilibrium constant may be determined at each temperature using the relationship;

$$K = Q/(Q_T - Q) \quad \text{Equation 5}$$

and the value of ΔH is then easily determined from;

$$\Delta H = \frac{Q_T}{A_T} \quad \text{Equation 6}$$

Such an analysis can be extended to more complicated reaction schemes and allows a complete thermodynamic description (determination of the rate constant as a function of temperature gives the activation energy, determination of the equilibrium constant gives ΔG and hence ΔS) of the reaction to be obtained.

3.3. Elevated RH Studies

Degradation rates of pharmaceuticals are often increased in the presence of water; for instance, water may induce a hydrolysis reaction, cause an amorphous sample to recrystallise by lowering its glass transition (T_g) temperature, cause deliquescence of crystals, result in the collapse of a freeze-dried 'cake', allow the formation of hydrates or act as an intermediary between two solid components. It is therefore important to know how a sample will react in the presence of water and, if necessary, reformulate or repackage the product to ensure there is no loss of potency upon storage.

IC can be used to study water mediated reactions *directly* because it is possible to design experiments where the RH in the sample ampoule can be accurately controlled (for most other analytical techniques, the sample must be stressed under an elevated RH prior to measurement). Conventionally, the RH in the sample cell is maintained using one of two techniques. The simplest method involves the placement of a small glass tube (a Durham tube, or hydrostat) holding water or a saturated salt solution directly within an air-tight ampoule containing the sample [46]. Saturated salt solutions will maintain a constant RH within a confined space at equilibrium [47]. The specific RH attained is dependent on the ambient temperature and salt used (for instance, a saturated solution of NaCl will give an RH of 75% at 25 ^{o}C). An alternative arrangement is to use RH perfusion [48]. In this case the RH of a carrier gas, flowing at a constant rate, is controlled as it passes through the sample ampoule. One such piece of apparatus is available from Thermometric AB (Järfälla, Sweden). In this system two gas lines are routed into the sample ampoule; one contains dry gas and one contains gas saturated with water vapour – proportional mixing of the two gas lines using mass-flow controllers allows a specific RH in the cell to be maintained.

It should be noted that both systems have advantages and disadvantages and, as always, care must be taken that unexpected or erroneous heat-flows have not arisen simply as a corollary of poor experimental design or execution. Most importantly, the effects of water evaporation and wetting of the ampoule need to be removed in order to analyse data quantitatively. For hydrostat experiments this is most conveniently achieved by using an equivalent salt solution in a reference cell, connected in opposition to the sample cell. RH perfusion experiments are usually conducted using a stainless steel ampoule containing a suitable quantity of an inert material as a reference and hence this correction is not possible. Perhaps the most important consideration is to ensure that the supply of water vapour is sufficient such that the measured sample response is not rate-limited. Rate limitation may occur if, for instance, the rate of evaporation of water from the salt reservoir is slower than the rate of water uptake by the sample (in the case of the hydrostat) or if the flow of carrier gas is too slow (in the case of RH perfusion). The hydrostat method gives rise to the generation and use of humidity within the measuring site of the calorimeter and hence much (but not all) of the wetting response for the sample is matched by the (almost) equal and opposite response for the generation of the humid air. Consequently the hydrostat method is very useful for studying samples that change following exposure to humidity, whereas RH perfusion is well suited to the measurement of wetting (and possibly subsequent changes if they are large enough to be visible in the presence of a large wetting response).

Control of local RH allows IC to be used to determine percent amorphous content in pharmaceutical powders. Many techniques, such as milling, grinding or drying, used in pharmaceutical manufacture induce the formation of amorphous regions in solid drugs and excipients. Although the amounts of amorphous material formed during processing are usually quite small, the mechanical nature of the processing techniques causes these amorphous regions to be located primarily on the surface of the particles. As such, the effects of the amorphous regions can be considerable as they exert a disproportionate control on the surface properties of the material. Above the glass transition temperature, amorphous materials tend to crystallise rapidly and, as there is a concurrent change in heat-capacity, the change can be followed using IC. Water acts as a plasticiser, lowering the T_g of the material, allowing crystallisation to be investigated at room temperature. The heat-output of crystallisation for a given material is directly proportional to its amorphous content and it has been claimed that it is possible to determine amorphous contents of 0.5% using microcalorimetry compared with a detection limit of 10% with conventional techniques such as X-ray diffraction [49].

Buckton and Darcy [50] have reviewed the use of hydrostats to study the response of amorphous lactose to changes in RH. A typical response shows two distinct regions; a small initial response reflecting absorption of water by the sample, followed by a large peak reflecting crystallisation of the sample and subsequent water expulsion. The onset of the second peak can be delayed by either increasing the sample mass or decreasing the RH. Decreasing the RH below a critical point leads to no crystallisation within a measurable time.

For lactose samples with low amorphous contents, the large crystallization peak is often observed to be split into two separate regions, referred to as parts II and III (part I being the small initial response). A result of this complexity is that the crystallisation enthalpy recorded for lactose using IC is significantly smaller than expected (for instance, it is lower than the melting enthalpy for lactose measured using DSC, which should be equal and opposite to the crystallisation enthalpy). Calculation of the crystallisation enthalpy using the area under the part II curve gives a value of around 32 Jg^{-1} whereas that calculated using both parts II and III gives a value around 48 Jg^{-1}. It has been suggested that part II corresponds to crystallisation and part III corresponds to mutarotation of α-lactose to β-lactose [51], although it should be noted that some samples that do not exhibit mutarotation also show two distinct peaks during crystallization, a good example being the crystallisation of salbutamol sulfate [52]. Since the contribution of mutarotation to the overall thermal response is not known, the best way to analyse lactose data is uncertain. However, the evidence from salbutamol sulfate crystallisation makes it likely that Peaks II and III are both part of the crystallisation response.

It has been argued that the smaller than expected enthalpy arises as a result of the (endothermic) desorption of significant quantities of water during the crystallisation phase [53]. Studies of the recrystallisation response of raffinose using IC have shown that the crystallisation enthalpy matches that of the melting enthalpy recorded by DSC [54]. Furthermore, a study of raffinose recrystallisation using DVS-NIR showed there was no significant desorption of water during the recrystallisation phase [54]. More recent work on lactose suggests that the isomeric composition of the crystalline component of the sample affects the measured enthalpy of recrystallisation by changing the ratio of α- to β-lactose formed (the heat of recrystallisation to the α-form being different from that to the β-form) [55].

The use of IC to detect levels of amorphous content, in micronised batches of an undefined active, of 0.5% w/v has been reported [56]. Aso *et al.* have studied the physical stability of amorphous nifedipine [57], reporting similar responses for the net crystallisation enthalpies at temperatures between 50-60°C over a range of relative humidities, and amorphous phenobarbital [58]. Other pharmaceutical materials that have been investigated using this methodology include amorphous cefditoren pivoxil [59], griseofulvin [60], albuterol sulphate [61] and acadesine [62]. The use of non-aqueous solvents to induce crystallization, and hence assess amorphous content of solid drugs, has also been discussed [63], using water/acetonitrile mixtures and erythromycin.

A principal disadvantage of the hydrostat method is that it allows the effect of only one RH upon a sample to be investigated with each experiment. Furthermore, the initial response from such an experiment is never observed as the open hydrostat must be sealed in the ampoule before being loaded into the calorimeter. Discounting the effects of friction and thermal equilibration from a conventional calorimetric experiment means that, typically, the first 30 min of data are lost, during which time a reaction may have progressed to a significant extent.

One approach to overcome this problem is to seal (with foil and wax) the sample in the hydrostat and place the hydrating liquid in the ampoule [64]. This affords the largest surface area possible for water evaporation (although this does not preclude the possibility that the rate of water evaporation is rate limiting) and allows the system to reach thermal equilibrium before any interaction is initiated. It also allows the internal surfaces of the ampoule and external surfaces of the hydrostat to be wetted before data capture. Once equilibrium has been achieved, the foil seal is broken (by means of a rod that is accessible from outside the instrument) and the sample and water vapour can interact.

Such a system has been used to investigate the behaviour of anhydrous ceftazidime, a drug that is known to form a mono-hydrate, under controlled RH [64]. The inherent thermal response characteristic of the apparatus was determined by running a number of control experiments. With no sample in the hydrostat and water in the ampoule the power-time plot showed a small exotherm and returned to the baseline within 1h. With water in the hydrostat and an empty ampoule (the equivalent of the 'standard' hydrostat experiment) the power-time trace showed a much larger endothermic response and did not return to the baseline for several days. Both traces reflected puncturing of the seal, friction from lowering and raising the pole used to break the seal and adsorption of water to the previously unexposed surfaces of the ampoule or hydrostat and it was clear that placing the sample within the hydrostat minimised the thermal response from the equipment.

The power-time trace obtained when the apparatus was used to study the hydration of anhydrous ceftazidime (with drug in the hydrostat) comprised three main regions, termed A, B and C [64]. Region A represents the mechanical effects of breaking the seal and wetting of the cell and sample and remained constant for each sample. Region B was seen to alter as the average particle size distribution of the sample varied, reducing from 3.95 h for 90-100 μm samples to 3.4 h for 45-60 μm samples, and was attributed to the absorption of water by the drug. Region C, a large exotherm, appeared to be slightly sharper with the lower particle size fraction, but the areas under each curve were the same within experimental error (45-60 μm – 42.3 Jg^{-1}, 90-100 μm – 40.5 Jg^{-1}). Powder X-ray studies showed that the sample underwent a change in crystal habit during this phase, suggesting that this exotherm corresponded to a change in the crystal structure of the solid drug as it formed the mono-hydrate.

Gas perfusion calorimetry has been used to measure the interactions between water vapour and a number of amorphous pharmaceutical solids (sucrose, lactose, raffinose and

sodium indomethacin) [65]. The power-time data exhibited general trends that aided an explanation of the effect of moisture content on the physical stability on the amorphous form at given storage temperatures. At some RH threshold (RH_m) the data showed a large increase in the energy of interaction between the water vapour and the sample that could not be explained by a phase or morphology change. Below RH_m water sorption/desorption was reversible; above RH_m hysteresis was noted and water-water interactions dominated the thermal response. Samples stored in an atmosphere below RH_m showed no evidence of instability after several months.

3.4. Stability Assessment of API-Excipient Mixtures

As discussed earlier, the principal methodology used to detect API-excipient interactions using IC is to compare the thermal responses recorded for the active and excipient alone with that obtained for an active-excipient blend. An unexpected heat-flow in the blend indicates some interaction is occurring. Such a qualitative analysis does not usually indicate the nature of the interaction, but it does allow a rapid assessment of the likely stability of a number of potential formulations. There are few reported studies where IC data of active-excipient blends have been analysed quantitatively, usually because of the complexity of the data, although this forms perhaps the most exciting potential growth area for the use of IC in the pharmaceutical sciences.

The well documented incompatibility between aspirin and magnesium stearate [23, 66] provides an ideal system to demonstrate the use of IC to investigate API-excipient mixtures. Under an RH of 100%, a binary mixture of aspirin and lactose shows no thermal heat response while a binary mixture of aspirin and magnesium stearate shows a large exothermic signal [67]. The heat output appears to follow zero-order kinetics and lasts for a period of several days. Analysis of the aspirin content in the ampoule after the heat-flow had returned to zero showed that the entire drug sample had degraded [67]. It has been suggested that the interaction between the drug and the metal carboxylate arises from a reduction in the melting point of aspirin, generating a liquid layer of drug on the surface of the magnesium stearate particles that accelerates degradation [66].

Interactions between a solid active and a range of excipients, including potato starch, α-lactose-monohydrate, microcrystalline cellulose (MCC) and talc have been investigated using IC, albeit between elevated temperatures of 60-80 ^{o}C [68]. Large exothermic heat responses were observed for mixtures of drug with MCC, potato starch and lactose, indicating these systems were unstable. A similar study has looked at the interactions of an active compressed into a tablet [69].

A good example of the use of IC to measure likely incompatibilities between actives in a multi-component parenteral formulation is shown by the data in Fig. **2**. In this case the system under study was a parenteral formulation administered to paediatric patients which contained three actives in one perfusion bag. By measuring the calorimetric power outputs of the three drugs alone a predicted response curve could be generated; this differed considerably from the actual response obtained from the three component mixture. In the absence of any supporting mechanistic data, these results suggest that there is at least one interaction between the components in the bag and that its use should not be recommended until further studies have been conducted.

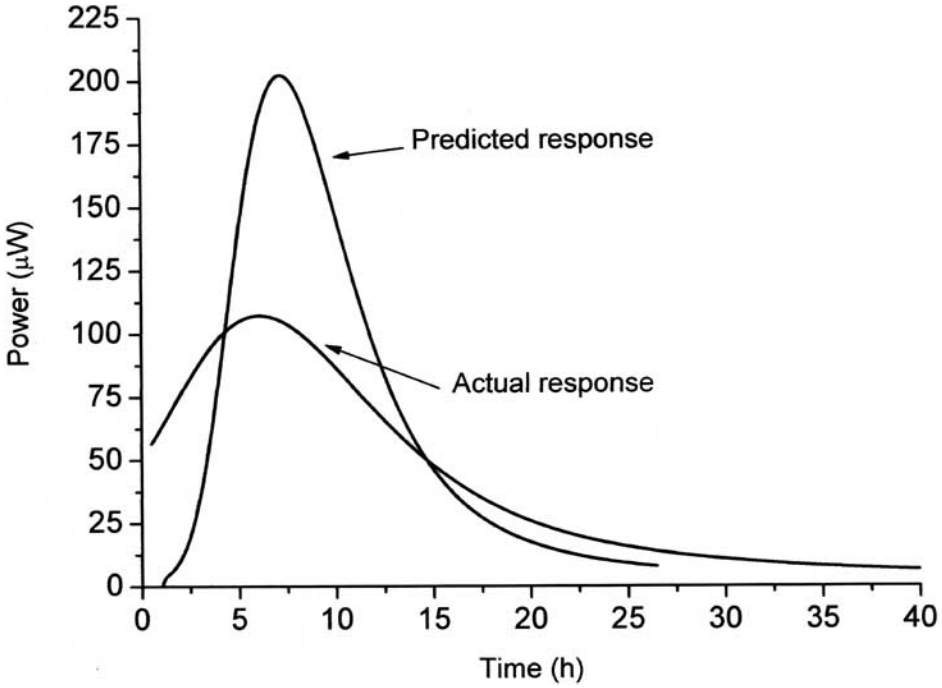

Fig. (2). Power-time data for the degradation of the components in a three-component infusion bag showing i) the predicted response generated by summing the power-time curves of the three drugs run individually and ii) the actual response of the admixture [Gaisford *et al*.; unpublished results].

Flow microcalorimetry has been used to study the interaction between heparin sodium and dopamine hydrochloride in two parenteral formulations [70]. A significant interaction between the drugs was noted when dextrose was included in the parenteral solution which was not seen in normal saline formulations.

The effect of menadione and prednisone on the physical stability of various microemulsions has been investigated by IC [71]. It was shown that neither drug influenced the stability of the formulation. Gaisford *et al.* [72] used IC for studying the swelling of PEG-based hydrogels in water. In these experiments a segment of dry hydrogel (xerogel) was immersed in water (1ml) and the heat response from swelling recorded. A typical trace showed a two-phase process, which the authors ascribed to the hydration of the polymer core and subsequent relaxation of the polymeric network. The break point time between the two processes was observed to reduce with an increase in storage temperature.

3.5. Future Developments

Heat is ubiquitous and with care it is already possible to study virtually any system using IC, so long as enough representative material can placed in the sample ampoule to give a large enough heat-flow signal to record over a reasonable time scale. However, it is currently difficult to use IC to study systems that degrade with slow rate constants and/or small enthalpy changes, samples that are scarce or very expensive (such as new APIs, for instance) or systems that give complex, multi-component heat-flow signals. Future developments therefore need to address these issues and may include developments in hardware design, coupled with new methodologies for data analysis and novel experimental design.

In terms of hardware design, one promising approach appears to be calorimeters based on solid-state devices (chip calorimeters). Chip calorimeters, such as the liquid nano-calorimeter manufactured by Xensor Integration BV, Netherlands, and the array-calorimeter developed by Vivactis, Belgium, have been shown to have a wide range of applications, including the detection of heat-flows in micro-litre liquid samples, temperature induced reactions in small solid-state samples and the study of solid-gas interactions [73]. The dimensions of the Xensor calorimeter (5 mm x 5 mm) mean its heat capacity is very small and it requires a very small sample volume. The reduction in heat capacity both reduces experimental run times and increases sensitivity and instruments based on such devices are currently commercially available (such as the Setsys calorimeter range, Setaram, France).

Advances are also being made in the analysis of complex data. Models based on reaction kinetics such as those described above allow the complete deconvolution and analysis of complex data sets but require some prior knowledge of the reaction mechanism in order to be confident in the fitting parameters returned. The recent development of a new analysis methodology, based on a chemometric approach may overcome this problem [74]. In a chemometric analysis, an algorithm is used to search for patterns in a matrix of data; this approach was successfully developed for the analysis of data recorded for systems studied using FTIR and the software package is commercially available [DiKnow Ltd., UK] and can be applied to IC data. The matrix of data requires three variables; these are easy to assign for FTIR analysis (time, wavelength and intensity) but are more problematic for IC data which have only two variables (power and time). However, the random noise and discrepancies inherent between repeat data sets is sufficient to form the third variable, which means that a chemometric approach can be adopted as long as a number of data sets (from the same sample) are available. Such an analysis returns plots of intensity versus time for each process present in a multi-process system and allows the recovery of first-order rate constants. However, as yet it is unclear as to the relationship between intensity and power, meaning that enthalpy values are not recoverable. The approach does allow an assessment to be made of the number of processes occurring with no prior knowledge of the system and it may be the case that an initial analysis of complex data using chemometrics, followed by an analysis using a specific kinetic model (which can be selected based on the results of the chemometric analysis) may be the best approach for the analysis of complex power-time data. A complete discussion of the principles of chemometric analysis and a review of its applications to date are given in a separate paper in this issue [75].

Novel experimental methodologies also offer a way of studying processes that are otherwise outside the scope of conventional IC techniques. Usually, such processes cannot be studied because they either occur with a small change in heat content or because they occur over extended periods of time and, hence, result in heat-flow signals that are indistinguishable from the baseline. IC measures the net heat-change that occurs during a process and it is possible to increase this value by linking the process of interest (which, by definition, has a small heat-change) to a reaction that has a large heat-change. If either the reactant or product is an acid or base, then it is possible to enhance the power signal by conducting the experiment in a buffer that has a high enthalpy of protonation. The formation (or loss) of a molecule of acid or base (low enthalpy) will cause the protonation or deprotonation of one of the buffer components (high enthalpy) leading to a measurable power signal that varies quantitatively with the study reaction. Because the enthalpy of protonation of the buffer is known, the enthalpy of the study reaction can be determined from the measured power-time signal. The phenomenon of linked equilibria has long been observed in isothermal titration experiments (and, indeed, has to be corrected for to ensure the correct enthalpy is attained) but has only recently been used to enhance the power signals for low enthalpy processes [75]. In principle, it should be possible to link any low enthalpy process with that of a high enthalpy process (even one that produces a 'cascade effect' such as the mechanism employed in a Geiger counter), allowing the study of all low enthalpy processes.

A different approach can be adopted if the purpose of the calorimetric experiment is to assess the quantity of a component that is present in a multi-component system rather than to determine the kinetics of its degradation. Recently an experimental approach was proposed that allows the analysis of ascorbic acid in foods to be determined using

batch calorimetry [76]. In this approach, a food sample is added to a solution containing ascorbate oxidase (an enzyme that selectively degrades ascorbic acid). This results in complete degradation of any ascorbic acid present within a few hours, giving a heat-change that is proportional to the ascorbic acid content. Such an approach magnifies the heat-flow deriving from the study reaction relative to those deriving from all the other processed in the system and potentially offers IC as a new, rapid method for conducting quantitative analysis of specific analytes.

SUMMARY

Isothermal calorimetry offers a combination of properties that makes it *uniquely* suited to pharmaceutical stability assessment. The detection sensitivity of modern instruments allows the investigation of systems directly under storage conditions, avoiding the assumptions inherent in elevated temperature studies. As long as a representative sample can be placed in the calorimetric vessel, the technique can be used to study virtually any system, regardless of the phase or number of phases present. Since heat is ubiquitous, there is no need for a sample to contain specific elements or to have a specific functional group, as there is for instance in spectrophotometric analyses, nor is there the need to prepare solutions, as is required for many chromatographic techniques. Furthermore, calorimetry is the only technique, which allows *direct* measurement of reaction enthalpies for both chemical and physical changes; in combination with data analysis using a suitable model a complete thermodynamic and kinetic description of a process can be obtained.

Isothermal calorimetry can be used for rapid screens of drug-excipient compatibility simply by comparing heat-flow responses recorded for the materials alone with that recorded for a binary mixture. Elevation of the local RH can be used to stress a sample. Any unexpected heat-flows in the binary system indicate a likely incompatibility; such a qualitative analysis allows the selection or rejection of a large number of potential formulations early in the development programme for a new pharmaceutical.

The development of analyses that can extract additional fundamental information from raw calorimetric data will undoubtedly lead to a more widespread use of the technique. If the sample is in solution then recent methodologies have demonstrated how kinetic, thermodynamic and even mechanistic information can be extracted from short-term power-time data. For reactions that follow, or are suspected to follow, first-order kinetics a simple plot of ln(power) versus time yields the rate constant. Knowledge of the amount of material reacting gives the change in enthalpy. Studying a system at different temperatures determines whether an equilibrium state is formed and, if so, allows the derivation of the equilibrium constant and, consequently, ΔG and ΔS. The analysis of solid-state samples presents more difficulties but fitting methodologies based on the Ng equation offer the potential to determine rate constants and give insights into reaction mechanism. Chemometric analyses allow information on the number of processes occurring to be gained with no prior information about the sample.

One of the next challenges is to extend these methodologies to allow the analysis of drug degradation or drug-excipient interactions directly within complex pharmaceutical preparations. A further challenge facing users of the technique is to get short-term calorimetric stability data accepted by national pharmaceutical regulatory bodies for demonstrating long-term product potency; one approach to this is to compare short-term calorimetric data with conventional long-term storage data and show that the calorimeter predicts accurately long term product stability. The future development of smaller, solid-state calorimeters, in combination with novel experimental methodologies and analysis techniques, can only serve to enhance the utility of IC in the pharmaceutical and biopharmaceutical fields.

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