The precipitation of candidate drugs on mixing DMSO stock solutions with aqueous buffers

By

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A thesis submitted to the University of Strathclyde in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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This thesis is the results of the authors' original research. It has been composed by the author and has not previously been submitted for examination which has led to the award of a degree.

Signed……. Date……

Acknowledgements

With thanks to;

My supervisor, Professor Peter Halling, for his time, guidance and support throughout the past three years.

My industrial supervisor, Dr Pierre Daublain, MSD, for taking the time to give an industrial opinion on the research.

Dr Darren Edwards, Univ. Strathclyde, for his knowledge and practical training on the Tecan instrument, and his comments on the research.

Dr Alan Kennedy and Professor Alastair Florence for their comments on papers drafts.

Lee Dowden (MSD Hoddesdon) for his help and training on the Biomek instrument.

Scott McKellar (formally Univ. Strathclyde) for assistance with the XRPD.

Craig Irvine (Univ. Strathclyde) and Ken Cameron (formally MSD Newhouse) for performing NMR analysis.

Iain Larmour (Univ. Strath) for assistance with the Raman instrumentation.

Jonathan Smith and Patrick Hole (NanoSight) for their technical advice on the instrument.

MSD and the University of Strathclyde for funding contributions.

My colleagues in TG 103 for their support and companionship.

Abbreviations, Acronymns and Definitions

Ami - amiodarone HCl

AFM – Atomic Force Microscopy

BSA – Bovine Serum Albumin

CAC – Criticial Aggregate Concentration

Clot - clotrimazole

DLS – Dynamic Light Scattering

DMSO – Dimethyl Sulfoxide

DSC – Differential Scanning Calorimetry

FeSSIF – Fed State Simulated Intestinal Fluid

FIA-MS - Flow Injection Analysis-Mass Spectrometry

g - earth's gravitational force, 9.80665 m/s^2 (see RCF)

G.I Tract – Gastro-Intestinal Tract

GSE – General Solubility Equation

HCl - hydrochloride

HPLC – High Performance Liquid Chromatography

HTS – High Throughput Screening

LogD – octanol/water distribution co-efficient

M – molar concentration, mols per litre

mm – millimetre, 1×10^{-3} metres

mM – millimolar, 1×10^{-3} mols per litre

MSD – Merck Sharp and Dohme

NMR – Nuclear Magnetic Resonance

NTA – Nanoparticle Tracking Analysis

ρ – density, g/cm^3

PCMCs – Protein Coated Microcrystals

pI – isoelectric point, defined as the pH at which a protein carries no net charge.

PLM – Polarised Light Microscopy

RCF – Relative Centrifugal Force, quoted as value x g

SEM – Scanning Electron Microscopy

'Shake-Flask' Solubility – the solubility value obtained from dissolving a crystalline solid in a given solvent system, at a given temperature, over an extended time period with agitation until the system is believed to have reached equilibrium.

TEM – Transmission Electron Microscopy

µM – micromolar, $1x10^{-6}$ mols per litre

µm – micrometre, 1×10^{-6} metres

µL – microlitre, 1×10^{-6} litres

UV – Ultra-Violet

XRPD – X-Ray Powder Diffraction

% - percent

Abstract

The precipitation of candidate drugs from dimethylsulfoxide (DMSO) stock solutions and buffer mixtures has practical importance within the pharmaceutical industry. At the drug discovery stage, mixing of drug DMSO stocks and aqueous buffer is a common procedure for many analyses, such as bioassay screening and some solubility screens. Precipitation from these mixtures, whilst a recognised issue, has little in the way of published work. This thesis explores in detail the precipitation of three poorly water soluble, commercially available drug molecules, namely amiodarone HCl, clotrimazole and tolnaftate. The work presented here assesses the process from a physical chemistry point of view.

Upon mixing the DMSO drug stock and aqueous buffer, all three compounds form particulates in the nanometre size region, which grow over time. The growth of these particles can be monitored using nanoparticle tracking analysis (NTA) and the NanoSight. We show that the presence of these particles can interfere with the results of 'kinetic solubility' measurements, depending on the exact analysis used. It is already known that these types of particles can interfere with bioassay results, causing false positives. Variables such as the mixing employed, the exact percentage of DSMO present in the samples and the concentration of protein present in the samples all affect the formation and growth of these particles. The results were correlated to high performance liquid chromatography (HPLC) measurements. It can therefore be concluded that there are several, controllable variables which can affect the precipitation of the three test compounds investigated here, and thus potentially the results of some discovery stage pharmaceutical screening assays.

Contents

1. Theory and Literature

1.1 Introduction

High Throughput Screening (HTS) is commonplace within the pharmaceutical industry, and is applied to many different processes, for example solubility measurements,[1,2,3] bioassays, [4] and salt and polymorph screens.[5] The need for HTS within the industry evolved from the sheer volume of compounds being synthesised, and the need to rapidly but efficiently evaluate their properties and activity, in order to streamline the number of compounds from thousands to the most promising few. HTS occurs early in the drug discovery process and involves miniaturization of the technique in question, allowing it to be automated, resulting in rapid testing and data production.[6,7] It is important to maintain low cost and use the minimal amount of compound, especially at the early stages of drug discovery.[6] Whilst speed is an important part of these screening methods, they must also be robust and reliable. Promising, possibly even lifesaving drugs could potentially be disregarded if the screening process is not efficient.

In order to maximise the efficiency of the HTS process, compounds are stored as stock solutions dissolved in dimethylsulfoxide (DMSO). This allows easier automation compared to dry compound storage, as liquid handling is readily performed by robots.[1,8] DMSO is a dipolar aprotic solvent with a large dielectric constant, meaning interaction with a molecule containing a dipole is favourable.[9] Since most drugs contain a dipole, the majority of pharmaceutical compounds will dissolve in DMSO, making it ideal for use in an HTS set up. Aqueous stock solutions are not used, due to the poor water solubility of many drug molecules. Different assays have different tolerance for DMSO, and generally the percentage of solvent is kept to a minimum to limit these effects. [4]

For both bioassays and solubility analyses at the early discovery stage, the stock solution in DMSO is introduced to an aqueous buffer system. As the aqueous solubility of many of the compounds under test is poor, precipitation may occur.

Low aqueous solubility is common, as drug molecules are optimised with a view to increasing their activity against a particular target, and the motifs introduced for this purpose tend to increase the lipophilicity of the drug.[10] In solubility assays, precipitation is indicative that the solubility limit has been reached, and is an anticipated phenomenon. In a bioassay setting, precipitation is undesirable, and results in a poor assessment of the compound's activity towards a particular target. There is a growing realisation within the industry that precipitation may occur during activity analyses.[4] Pope-Burke *et al* note that whilst long term stability of the aqueous solutions used in assays is not a relevant issue, the initial solubility and stability can be.[11] The concentrations used in HTS are low, typically in the µM range. Literature on the crystallisation of organic compounds, which has been written from a preparative viewpoint, generally deals with higher concentrations. Despite the implications for HTS, little is known about the factors which affect crystallisation and precipitation at these low concentrations.

1.2 Theory

Solubility is a widely studied topic, particularly in the pharmaceutical industry. Since aqueous solubility is directly related to absorption and bioavailability *in vivo*, it is an important physical property of drugs which are intended to be administered orally.[12] Oral dosing remains the most popular method of drug delivery, with advantages such as self administration and low cost of manufacture.[13] Crystallisation has also been studied extensively, and theory and principles of crystallisation are widely available in literature. Both crystallisation and solubility underpin the work in this project, with an understanding of the theory in the literature crucial in order to determine whether the systems which will be studied here behave as would be expected. These two processes will now be discussed in turn.

1.2.1 Solubility

The solubility of a compound is the maximum amount of solid that can be dissolved in a certain volume of liquid, under known solution conditions. The 'true' thermodynamic solubility of a compound is regarded as that of the most stable solid form which has been achieved under equilibrium conditions i.e. the amount dissolved does not vary over time, and is equal to amount left molecularly dissolved in solution after a precipitation has reached full completion. From the perspective of the pharmaceutical industry, the aqueous solubility of a compound is directly related to absorption and bioavailability *in vivo*.[12] The rate at which a compound dissolves is known as the dissolution rate, and can be described by the Noyes-Whitney equation;

$$\boldsymbol{J = kA(Cs - C)}$$

Equation 1

Where;

J is the dissolution rate

k is a constant consisting of the diffusion coefficient divided by the diffusion length

A is the surface area of the solute

Cs is the solubility

C is the concentration of solute in the medium

The dissolution rate is related to the solubility of the compound.[14]

Many factors affect the experimental solubility value of a molecule, and can be broadly grouped into three categories;

i. Experimental conditions used

The medium used, the temperature, and the time that the sample has been left to equilibrate for all affect the solubility value seen, and it is important to note these conditions when quoting a solubility value. Once the equilibrium solubility has been reached, time does not affect it – however whether or not equilibrium is reached within the time frame of the experiment will affect the result obtained. Any filtration processes performed, and even the type of instrument used to analyse the sample can also affect the result of a solubility experiment.[12] In addition, the mixing process employed contributes. The addition of salts to the medium can have a 'salting in' or salting out' effect, resulting in a higher or lower solubility, respectively.[9,14]

ii. Properties of the solute

Structural properties of the molecule, such as the pKa, lipophilicity and size, can also affect the solubility, and modifications to the structure can change the solubility. The pKa of the molecule, and the pH of the solution, have a large impact on the solubility seen. In general, the ionised form of the molecule is more soluble than the neutral form (intrinsic solubility), and so the solution pH should be stated when

quoting solubility values. At pH = pKa, 50% of the molecules in the solution are ionised, and the solubility is increased compared to the intrinsic solubility.[14] The solubility of salts is also dependent on the counter ions present. Modifications to improve aqueous solubility include addition of ionisable groups (e.g. –COOH), or addition of hydrogen bond donors and acceptors (e.g. $–NH_2$, -OH). Alteration of the structure of the molecule, and the pH, affect the equilibrium solubility value (due to a change to a different but related molecule, or due to changes in the percent of the drug ionised).[12]

iii. Physical form of the solute

The physical form the solute is presented in, e.g. whether crystalline or amorphous, and what polymorph is used, affects both solubility and dissolution rate. Crystalline solids tend to have lower solubility values than their amorphous counterparts.[15] The physical state can also include the particle size of the solid, and whether a salt form of the drug is used. It should be noted that the physical state in which the compound is introduced into the medium affects the dissolution rate and the apparent solubility of the compound.[14] The apparent solubility is the solubility of a solute in a form which is not the most thermodynamically stable, or has been altered in some way, for example by particle size reduction.[14] The equilibrium solubility is not affected. Although amorphous particles tend to be more soluble than crystalline ones, if left for sufficiently long enough, the material will convert to its most stable form, and the solubility approaches that of the thermodynamic value. Use of different crystalline forms can only increase dissolution rate and give a temporary change in solubility, however these effects may be useful in the pharmaceutical industry to increase absorption within a certain time period, for example to allow absorption within the gastro-intestinal (G.I.) tract to occur.[14]

Particle size reduction can be achieved by micronisation, milling or micro-crystallisation, and increases the dissolution rate and apparent solubility of the compound. From the Noyes-Whitney equation, it can be seen that an increase in

surface area (per unit volume) due to smaller particles would result in an increase in dissolution rate. From the Kelvin equation;

$$\boldsymbol{RTln\left(\frac{S}{S_0}\right) = \frac{2\gamma V}{r}}$$

Equation 2

Where;

γ is the surface energy of the solute

V is the molecular volume of the solute

S_0 is the solubility of an infinity large particle

R is the universal gas constant

T is temperature (Kelvin)

R is particle radius of the solute

S is the solubility of the solute

the apparent solubility of the compound also increases as particle size (radius) decreases. The processes used to reduce particle size can also result in imperfections within the solid - it is less 'crystalline' than before, is not the most stable form of the solid, and therefore only it's apparent solubility is altered.[14]

Another common way of increasing solubility and dissolution rate of a drug is salt formation. Again, the equilibrium solubility is not affected - it does not matter whether the compound is introduced as the salt or the free acid/base, the solubility achieved by both is the same at the same pH. The salt form will, however reach this value faster and can achieve supersaturation, whereas the free acid/base would not normally dissolve to supersaturation. This means, for example, that the salt form of a drug could fully dissolve to its maximum solubility or even achieve and maintain supersaturation, prior to transit through the gastro-intestinal (G.I.) tract, allowing

absorption to occur. The free acid/base, on the other hand, may not even reach maximum dissolution during that time.[12]

1.2.2 Crystallisation

The process of crystallisation can be broken down into three main stages;

i. The formation of a supersaturated (non-equilibrium conditions) solution
ii. Nucleation
iii. Growth [16]

These will now be briefly described in turn.

i. <u>Supersaturation</u>

Once the maximum amount of solute has been dissolved in a solvent, the solution is said to be saturated, and is in equilibrium with the solid phase. It is possible, e.g. by evaporation or cooling, to achieve a solution where there is more dissolved solute than would be seen under equilibrium conditions. This is known as supersaturation. In relation to screening, supersaturation can occur when the stock solution in DMSO is added to the aqueous buffer – it is possible to achieve a higher concentration of solubilised drug in the (mainly) aqueous solution than would be seen under normal conditions. The relationship between saturation, supersaturation and crystallisation can be depicted as follows;

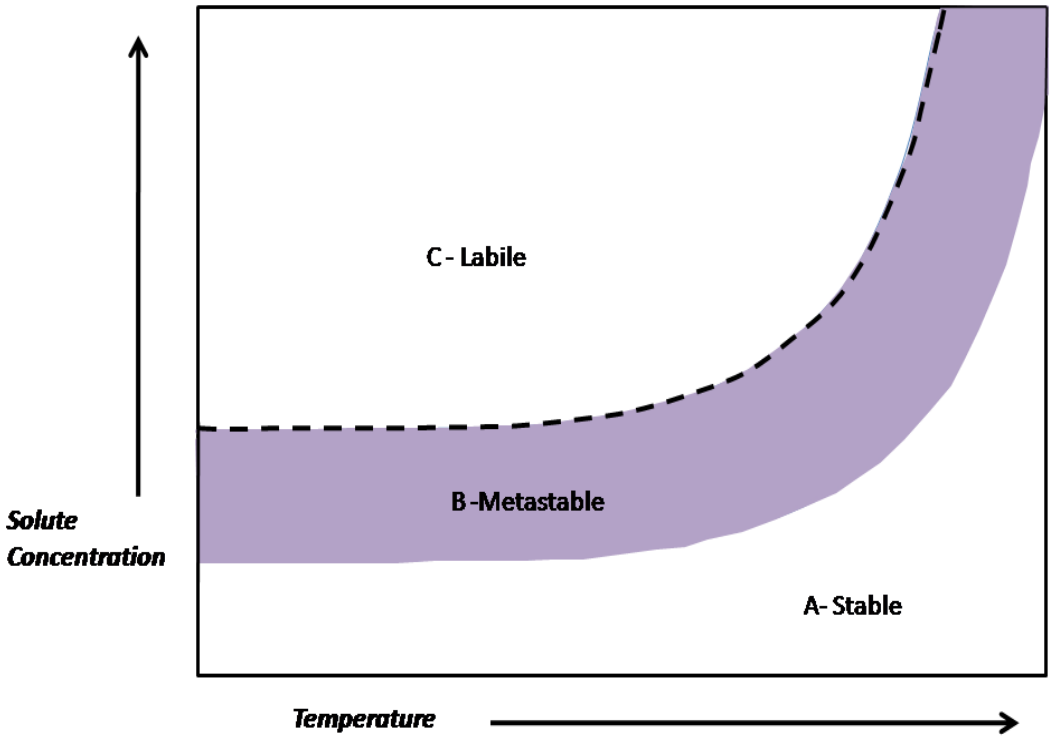

Figure 1: Supersaturation diagram

In region A, the solution is undersaturated with respect to the solute. The line between regions A and B represents the saturation solubility. Both regions B and C are supersaturated, however there are differences between the two in terms of nucleation. In region B, no spontaneous crystallisation can occur, and only seed crystals can grow. Spontaneous nucleation cannot occur until Region C.[9] The terms 'metastable' and 'labile' were introduced by Ostwald, who was the first to specify these conditions exactly.[17]

ii. Nucleation

A nucleus can be defined as 'the minimum amount of a new phase capable of independent existence'.[16] Experimentally, the existence of a critical nucleus was first demonstrated by Oswald.[17] Erdemir *et al* compare and contrast two nucleation theories, classical nucleation theory, and the two step model of nucleation. Classical

nucleation theory is the simplest and most commonly used theory to explain the process of nucleation – here, a critical radius must be reached before it is energetically favourable for the nucleus to remain as a cluster and grow rather than re-dissolve. There is a competition between the surface free energy, which favours dissolution, and the volume free energy, which favours growth – only at nuclei of a certain size will growth become possible. This theory assumes that the nuclei are already ordered in a manner that is identical to the bulk crystal, and that the nucleation rate is time independent. It also assumes that growth occurs by addition of one monomer at a time. The theory has a number of shortcomings, for example it cannot predict absolute nucleation rates, no information on the structure of aggregates or the pathway from solution to solid is provided, and the only criterion of whether aggregates form nuclei or not is radius. These shortcomings, along with experimental discrepancies, have prompted the development of other theories, such as the two step nucleation theory. This theory proposes that nucleation is a two step process, with the first step involving the formation of a highly disordered liquid droplet, then re-arrangement of the droplet to a crystalline nucleus beyond a certain critical size. Here, the second, crystalline forming step is the slow, rate determining step. Computational, theoretical and experimental studies are described in the work of Erdemir *et al* which support the two step nucleation model.[16]

Factors which affect nucleation include impurities present in the system, temperature, the amount of supersaturation in the system, and mechanical shock e.g. sonication, agitation. It has been found that agitation can induce nucleation at lower degrees of supersaturation compared to a solution left unperturbed, however it is thought that there may be circumstances where agitation can in fact lower the tendency to nucleate. Impurities can increase or decrease nucleation rates, depending on the system.[9]

iii. Growth

Many growth theories exist, and can broadly be categorised into three groups; surface energy theories, diffusion theories and adsorption layer theories. Surface energy theories have the basis that the shape of a growing crystal is that with the minimum surface energy – Gibbs suggested that 'the total free energy of a crystal in equilibrium with its surroundings at constant temperature and pressure would be a minimum for a given volume'. The main limitation of this type of theory is its inability to explain the effects of supersaturation and solution movement on growth, and so surface energy theories are rarely used.

Diffusion theories presume that material is deposited onto the surface of the crystal continuously, at a rate proportional to the concentration difference between the point of deposition and the bulk solution, and initial theories regarded crystallisation as the inverse of dissolution. This is not the case, as most solids will dissolve faster at the same temperature and pressure conditions than they will crystallise. Later modifications to the theory suggested that a two step process occurred; a diffusion process where molecules are transferred from the bulk to the solid, then a rearrangement process where the molecules arrange themselves into the crystal lattice. These processes are driven by differences in concentrations.

Adsorption layer theories suggest that crystal growth takes place by layer-by-layer adsorption onto the surface, arising from imperfections. The theory is based on thermodynamic reasoning. When the units of crystallising substance arrive at the crystal face, they do not immediately form part of the lattice, but instead lose one degree of freedom and are adsorbed loosely onto the surface. This 'third phase' is in dynamic equilibrium with the bulk solution. Some imperfections in the layer-by-layer adsorption are seen, and these dislocations, the most important of which is the screw dislocation, cause steps to be formed on crystal faces, promoting growth.[9]

After formation and growth of particles, other processes can occur, particularly in systems where fast crystallisation has occurred. Small particles have a tendency to

aggregate in solution and form clusters. This is most common at high supersaturation.[9] This has been discussed by McGovern *et al* in relation to the effect of aggregates on bioassays results (see section 1.3.4).[18, 19] Particles can also age in solution, by both ripening and phase transformations. As discussed previously, smaller particles are more soluble than larger particles – in a sample of particles in solution, the smaller particles tend to dissolve, and then the solute re-deposits itself onto the larger particles, which is more energetically favourable. This process is called Ostwald ripening, and has the overall result of increasing the size of the larger particles and decreasing the size of the smaller particles.[14] In theory, the particle size distribution of a sample undergoing ripening should change over time to become more monodisperse, however the distribution will initially become more polydisperse until ripening is completed. Ripening tends to occur at low supersaturation.[9] There is also the possibility of the initial solid being a metastable (e.g. amorphous) precipitate, and then phase transformation to the most stable form over time occurs. This follows the principle of Ostwald's rule of stages – an unstable system does not always transform into the most stable state, but the one which is closest in energy to its own. This means that the form which precipitates fastest is not always the most thermodynamically stable form.[9]

1.3 Literature Review

1.3.1 Overview of literature explored

As discussed in the introduction, the precipitation of drugs during screening upon mixing DMSO stock and aqueous buffer is an important issue. Much of the literature on this topic deals with practical steps to minimise precipitation[4] and the effects of any small particles on the results of an enzyme assay,[18-26] rather than fully investigating the underlying process of solid formation or parameters affecting precipitation. There is also literature available on the stability of the DMSO stock solutions,[27-34] which could also affect the result from the assay. Kinetic solubility assay methods, where precipitation is utilised as an indicator of the end point of the assay, are also explored within the literature.[2, 3, 10, 15, 35-38] Since the protocols for both activity and solubility screening have similarities in both concentration and use of DMSO stock, the methods used for solubility screens and observations from such experiments are useful in obtaining information on the factors which affect formation of solid at these low concentrations.

1.3.2 Storage in DMSO and its shortcomings

DMSO is regarded as a 'universal' solvent, and will readily dissolve most hydrophobic drug compounds. It is miscible with water, has a reasonably high polarity, low volatility and is relatively cheap and non-toxic.[32] It is also ideal for use in biological assays as it does not interfere with the biological components in low concentrations (typically 1-5% by volume), unlike other organic solvents.[30] Dry DMSO cannot form hydrogen bonds with itself as it only has acceptor capability, and so solvent cavities are easily formed around the drug molecules. Cavity formation cannot occur in water due to hydrogen bonding between the water molecules, which the drug molecules cannot disrupt. DMSO and water have very pronounced interactions, which is the drive for DMSO's hygroscopicity. In a DMSO/water mixture, the organic solvent acts as a hydrogen bond acceptor and forms hydrogen bonds with the donor water molecules, giving a more structured

solvent compared to DMSO alone. This makes it harder for cavities to form, and so the solubility of a molecule is decreased compared to dry DMSO. At 33% water (by weight) in DMSO, the structure of the solvent is more ordered than that of pure water.[29]

Typically, stock solutions are stored at concentrations of 10-30 mM,[4,12] with the mother stock solution being diluted as required for subsequent analyses. Whilst DMSO has several advantages, it has been reported that 10-20% of compounds are in fact insoluble in DMSO.[4] There are also concerns over the uptake of water into the DMSO stock solution which can result in the precipitation of the drug from the stock solution,[34] changing the concentration of the solubilised compound, and any further dilutions will therefore be erroneous.[12] Stocks are usually stored frozen at low temperatures to minimise degradation, with Blaxill *et al* demonstrating that compound integrity (affected by either precipitation or degradation) is related to both temperature and storage time. Based on extrapolations from experimental stability results (from 530 structurally diverse samples) at room temperature and 4 °C, they recommend samples stored at 4 °C were stable for 3 months, and samples stored at -20 °C stable for up to five years.[33] Kozikowski *et al* used statistical analysis of experimental FIA-MS data to calculate the probability of observing the correct compound at a given time point. They concluded that after 6 months storage at ambient conditions, there is still a good chance (83 %) that the mass spectrum matches that produced at time 0. The work done here looked at around 7200 compounds stored at room temperature for a year.[28] Cheng *et al* performed an accelerated stability study at 40 °C, and from the results calculated that compounds stored in water free (under inert gas atmosphere) DMSO are stable (≥ 80 % compound retention) for up to a year at room temperature. The use of low temperatures to store the DMSO stock solutions has also been explored – this however lowers the compound's solubility, and can result in the same issue as water uptake i.e. precipitation.[4] Both Blaxill *et al* and Ilouga *et al* have reported that lower storage temperatures lead to a reduction in precipitation/degradation compared to higher temperatures – if precipitation is the main cause of issues in

the stock solution, it would be expected that lower temperatures would exacerbate the issue as the solubility would be lower – it appears that lower temperatures have minimised water uptake in these cases. Cheng *et al* also state the importance of controlled humidity when storing the stock solutions.[34]

Frozen stock solutions are put through a series of 'freeze-thaw' cycles, again increasing the risk of the compound 'crashing out',[27,29] with Oldenburg *et al* reporting a synergistic effect between water uptake and freeze thaw cycles. Cheng *et al* report no precipitation or degradation after 11 freeze-thaw cycles.[34] As well as avoiding water uptake and minimising freeze thaw cycles, other practical measures to avoid precipitation include the use of sonication post freeze thaw,[29] and the use of an additive to retard crystallisation.[39] Lowering stock solution concentrations is also a recommendation, with the optimum concentration reported to be 2-5 mM.[11] Results of studies of compound storage and stability are difficult to compare due to the differing conditions used, including different experimental/statistical analysis methods used and different criteria/definitions of 'stable'. These all tend to be based on the authors' current practice.[31] This means that whilst there are several papers on this issue, none give a definitive answer with regards to which storage conditions are optimum, and apparently contradictory results and time limits are seen. There also seems to be a balance which needs to be achieved between the use of low temperatures to minimise degradation, and ensuring that no precipitation is occurring due to low solubility at decreased temperatures, whilst all the while minimising water uptake. Water uptake by DMSO is a major issue, with all authors agreeing that increased water content enhances precipitation and degradation in the stock solution.

1.3.3 Utilisation of the DMSO stock solution – bioassays

The DMSO stock solution is used as appropriate in a number of different analyses, for example 'kinetic solubility' assays and bioassays. In both analyses, the stock is added to an aqueous buffer system, with the pH used dependent on the analysis. The percent DMSO used in bioassays is dependent on the individual bioassay tolerance for DMSO, although overall it has limited biological effect at low concentrations.[30] The small percentage of organic solvent can also help solubilise the drug in the aqueous media, by disrupting the hydrogen bonding network in water. Since this would not give a true 'aqueous' solubility, the percent DMSO in solubility screens is kept low to try and minimise this solubilisation effect. Even at the low concentrations used for drug screening, there are significant differences between the solubility of the drug in DMSO and that in an aqueous solution. [11]

The addition of the DMSO stock solution to the aqueous system can cause supersaturation, which can be desirable, but can also lead to precipitation of the drug. In a bioassay setting, if the drug precipitates upon addition of the aqueous media due to low solubility, its activity against the target cannot be properly assessed due to the fact that some of the drug added initially is not present in solution. This is demonstrated by the fact that low solubility libraries have lower HTS hit rates.[4] It is estimated that around 30 % of discovery compounds have aqueous solubility lower than 10 µM, which is within the concentration range typically used in screening (although the exact concentrations used depend on the assay in question).[40] There is also the observation that activity can vary greatly depending on the exact assay composition.[12] Popa-Burke *et al* concluded that the solubility of the drug in the assay medium is more of an issue than stability – the plates are kept for around 24 hours on average. The group also note that the actual amount of drug present in the assay samples is significantly different to that expected, even at µM concentrations.[11]

A number of recommendations have been made in the literature to either prevent precipitation or to re-dissolve any solid material formed. [4, 29] These include performing serial dilutions in DMSO rather than diluting with aqueous buffer, using kinetic solubility data to adjust results from bioassays, reducing screening concentrations, [4] and the use of in-well sonication to re-dissolve any solid material formed. [29]

1.3.4 Aggregation based non-stoichiometric inhibition in HT activity screens

In 2002, McGovern *et al* reported that a number (15) of diverse compounds, introduced into aqueous media via DMSO stocks, all inhibited a series of unrelated model enzymes. The compounds did not behave as stoichiometric inhibitors, and in a screening context, they would give a false positive result. Non-stoichiometric inhibition is an undesirable characteristic, and means the compound is demonstrating non-classical interaction behaviour with the enzyme. The group found that these compounds all had common, distinctive behaviours – inhibition was time dependent, there was a change in the IC_{50} value when enzyme concentration was increased, the compounds all had unusually steep Hill curves, and showed sensitivity to detergent.[22,20] All of the compounds formed aggregates (diameter 95 – 400 nm) in solution, which were detectable by DLS.[18]

It was proposed that these aggregates were responsible for target inhibition. Several properties of the aggregates themselves were investigated – the aggregates were 'destroyed' upon the addition of detergent, and formed at a 'critical aggregate concentration' (CAC). TEM images of the aggregates were obtained, showing spherical particles. Further investigation of these aggregates suggested micelle like behaviour, due to the CAC, the fact that the dissolved content remained constant above the CAC, and that a fairly constant size distribution was obtained, despite increasing total compound amount.[26] Note that whilst this is consistent with micelles, it is also consistent with precipitation. None of these papers mentions the equilibrium solubility of the compounds used, or if the concentrations used are in

fact above this. The authors however, state that they do not think the aggregates are an early stage of precipitate, due to the ease with which the aggregates re-dissolve upon dilution to below the CAC. They refer to the difficulty at driving precipitated organic material back into aqueous solution, and reason that since dissolution of the particles after dilution is rapid, the particles are not precipitate.[26] Another way to think of this is that, typically, solution in contact with an organic precipitate is likely to be saturated, and so it is thermodynamically unfavourable for the solid to re-dissolve. In the case of the aggregates, dissolution only occurs when the compounds are diluted to below the CAC i.e. below the solubility limit of the aggregates. Since the particles are in the nm region, their solubility will be slightly higher than larger particles due to higher surface area. The small size of the particles also allows for much faster dissolution compared to larger particles. Compounds which formed these aggregates were present in both screening libraries and marketed drugs.[19]

Of major interest was exactly how these aggregates inhibited the enzyme. Denaturation was ruled out, however some small scale unfolding was found.[24] It was hypothesised that the enzyme was sequestered on the aggregate surface, with Coan *et al* demonstrating that there is more than enough surface available on these aggregates to accommodate the inhibited enzyme.[26] Exactly what interactions govern this surface adsorption is as of yet unknown, though they are likely to be of a hydrophobic nature. The phenomenon has some features in common with PCMC formation – both processes involve the surface adsorption of proteins to small molecule clustered species.[41] Several differences are of course also present (the solubility of the protein in the respective media used and the crystalline form and size of the coated clusters being the main ones), but a similar overall effect is seen. Several attempts have been made at both prediction of compounds which would form these aggregates [19], and high throughput methods of detection of aggregates . From a prediction point of view, Seidler *et al* found that whilst structural similarity was not related to aggregating capability (based on 5 related azole antifungals), hydrophobicity was. Physical properties such as solubility and clogP values were

fairly reliable indicators, however they were still not accurate enough to classify compounds definitively (clogP cut off applied – 81% correctly classified from 111 compounds, solubility cut off applied - 87% correctly classified from 111 compounds). Using more complicated prediction methods incorporating molecular descriptors, the success rate was increased to 94% of 111 compounds correctly predicted as aggregating or non-aggregating.[19] From a detection point of view, DLS has been used directly to detect these aggregates, as have TEM/SEM – however these two microscopy techniques cannot be used in a high throughput manner. Not mentioned in bioassay screening literature, but of potential use are the light scattering detectors used in kinetic solubility type analysis. These can be utilised in multi-well plates, and could potentially offer some use here for detection purposes in a high throughput manner. These methods are said to have a lower limit of 20 uM.[35] More indirect methods of determining aggregators include testing for detergent and enzyme concentration sensitivity. Habig *et al* found that some non-aggregating compounds could be sensitive to detergent, and that sensitivity to enzyme concentration was not only a more reliable indicator of an aggregating inhibition, it also eliminated non-stoichiometric inhibition in the same way as the addition of detergent would.[21] Kerns *et al* recommend lowering screening concentrations within bioassays to e.g. 5 µM, where aggregation is less common.[12] Owen *et al*[42] explored the effect of aggregate formation of anti-cancer drugs within cell culture. They found that three compounds have the ability to form colloidal aggregates in cell culture media and that efficacy of the aggregates is lowered. The presence of aggregates in cell based assays is therefore likely to results in a false negative effect – interestingly this is the opposite of the false positive effect that aggregates have on enzyme based assay.

Whilst several papers have documented thoroughly the effect of these aggregates on the results of activity assays, little is known about the aggregates themselves. Their physical form, behaviour under assay conditions, kinetics of formation/growth and thermodynamic stability in assay medium are all unknown.

An interesting follow on from aggregation *in vitro* affecting bioassay results is the possibility of it occurring *in vivo* and directly affecting drug absorption into the body. Frenkel *et a*[43]*l* looked at this possibility by studying a series of non-nucleoside reverse transcriptase inhibitors (NNRTI's, used as anti- AIDs drugs) and correlating their adsorption in rats and humans (from blood plasma studies) with their aggregation properties. These drugs tend to be very potent and extremely hydrophobic. In media which mimicked the *in vivo* gastrointestinal environment, it was found that the compounds formed aggregates which were measurable by DLS. The aggregating compounds fell into two groups; those which formed aggregates 30-110 nm in radius and those which formed aggregates >250 nm in radius. Those forming smaller sized aggregates showed greater absorption, with those forming bigger sized aggregates having poorer absorption behaviour. If a compound could maintain these smaller particle sizes for the duration of the GI tract transit time (and while encountering continuous pH increase), it is likely more drug will be absorbed. The particle size and absorption data suggest that this class of drug is taken up in the absorption tests in aggregate form, and it is suggested that this may occur for many other hydrophobic compounds where aggregates are formed. Whilst this work provides a basis for comprehending aggregate formation and absorption, many factors are still unknown, such as the sustainability of these aggregates as they move along the GI tract, and exactly how the aggregates themselves are absorbed.[43] Other work in this area includes that of Doak,[44] who found that 6 of 22 compounds which formed aggregates in buffer also had the capability to form aggregates in simulated intestinal fluids (FeSSIF). The compounds were tested at drug concentrations typical of these seen in vivo in the intestine. DLS measurements demonstrate the ability of some compounds to form colloidal aggregates in media mimicking *in vivo* conditions, again suggesting the possibility of these compounds being absorbed in this form. It was hypothesized that the drug is able to assume surfactant like properties under physiologically relevant conditions, which in turn leads to the aggregation effects attributed to their high potency.[45] Coan *et al*[25] looked at another aspect of the *in vivo* environment, namely high

protein concentrations, and found that aggregates are not disrupted by milligram per mL protein concentrations. This adds further evidence to the possibility of aggregation *in vivo*, for at least some compounds. The possibility of in vivo formation and subsequent absorption of aggregates is a very interesting hypothesis, which will no doubt be investigated further in the literature in the coming years.

1.3.5 Utilisation of DMSO stock – kinetic solubility assay

Analyses which evaluate a compound's solubility whilst starting from DMSO stock solutions are generally known as 'kinetic solubility' assays. A compound's kinetic solubility can be said to represent the maximum solubility of the fastest precipitating form, and is used in the early stages of discovery to give an indication of potential solubility issues; its thermodynamic solubility is the maximum solubility of the most stable form, and is regarded as the true solubility of the compound. Kinetic solubility measurements are generally regarded as sufficient at the early stages of the drug discovery process to give an idea of aqueous solubility and are indicative of any potential solubility issues early on. They are also developed to mimic the procedure employed during screening bioassays, with the solubility results used to aid in interpretation of HTS results.[38] They are rapid, automated and used to give an early indication of potential solubility issues.[15] Thermodynamic measurements are typically performed during the development stages. These solubility measurements do not use compound pre-dissolved in DMSO – the solubility is determined from crystalline solid which has been left to equilibrate with a given solvent over a period of time. These solubility assays are therefore initially affected by the dissolution rate of the compound, until equilibrium has been achieved – for kinetic measurements, the compound is already dissolved in DMSO, so this effect is not important in the assay. Thermodynamic solubility assays also use more compound compared to the kinetic assay, which at a discovery stage is important as there is not a vast quantity of drug available.[8] The measured thermodynamic solubility and kinetic solubility of a drug can be very different, with the kinetic solubility being either equal or higher – for the majority of compounds

analysed by Hoelke *et al*,[15] the kinetic solubility values are higher compared to thermodynamic measurements. The difference can occur due to many factors – as stated before, the kinetic solubility can be said to be the maximum solubility of the fastest precipitating form, which may have a different solubility from the most stable form. Bard *et al* [38] also found differences between kinetic and thermodynamic solubility values, and attributed these to both the ability of certain compounds to remain supersaturated when starting from DMSO stocks, and a co-solvent effect of DMSO. The DMSO present in the kinetic solubility assay may help solubilise the drug, and although the percentage is generally kept to a minimum, there still may be some effects. Bard *et al* demonstrated that while co-solvent effects due to DMSO were fairly general amongst a set of test compounds, the ability to remain supersaturated was more compound specific. The work looked at the effect of shaking intensity on the kinetic solubility value obtained, and determined that less vigorous shaking resulted a in supersaturated solution with less tendency to nucleate. This effect was more pronounced for low solubility compounds. Only kinetic solubility measurements were performed, with no analysis of the precipitate. Chen *et al* found that solubility enhancement by DMSO was highly compound specific, and, for poorly soluble compounds, increasing the % DMSO present can give significantly higher results.[46]

There is also the issue of the time that the experiment has been performed over – typically thermodynamic assays are performed over extended time periods to allow the system to reach equilibrium. Kinetic assays tend to be shorter, although some work has looked at prolonging the experimental time periods used. It was found that extension of the incubation period resulted in solubility values closer to the thermodynamic solubility value.[38]

Two widely used, general approaches of performing kinetic solubility are used; precipitation, separation of the suspension and finally analysis of the supernatant/filtrate content, or determination of the concentration at which precipitation first occurs. Measurement of the dissolved content involves either

filtration or centrifugation to separate the liquid and the solid, after a period of incubation. UV [35, 15] or HPLC [15, 47] analysis is then performed. Using the alternative approach, turbidity [10] can be utilised to detect the presence of precipitate, indicating the solubility limit of the compound – nephelometry [1,2, 15] is also used in a similar way. Analyses are typically performed in HTS 96- or 384 well plates. For a kinetic solubility assay determined by nephelometry, the stock solution is added incrementally to an aqueous buffer until precipitation occurs. This can, however, result in varying final % DMSO between different drug samples. Alternatively, an excess of compound can be added such that precipitation occurs, and dilution performed until the detected precipitate dissolves. This approach allows the percent DMSO present to be kept constant.[2] Kinetic solubility measurements using nephelometry typically determine the drug concentration at which light scattering become significantly different to background scattering – this is a similar concept to the CAC described in the previous section. Nephelometric kinetic solubility methods have been quoted as a having a lower limit of 20 μM - compounds with kinetic solubility values below this value cannot be accurately assessed.[15]

Alelyunas *et al*[8] have described the removal of DMSO prior to addition to the buffer, to give a solid for use in solubility measurement while still utilising the liquid storage of the drug. This approach gives results which are closer to the thermodynamic solubility, whilst still retaining the advantages of having the compound dissolved in DMSO, and a 'solid state normalisation' is seen. This suggests that the solid film formed after removal of DMSO resulted in the same solid state of the material being produced every time, and would remove any variation in the measurement from the formation of different solid forms. The work also suggests that for most of the compounds studied, equilibrium is reached during 24 hours of stirring – if this is the case, the solubility value seen should be independent of the starting form of the compound. This is of course assuming equilibrium has been reached between both the solid and liquid phases (dissolution now constant) and between different solid forms (conversion to most stable polymorph has been completed). The paper also notes that different methods of stirring and mixing give varying solubility results for

a model compound, glyburide. Replicate analyses were performed using both the dried DMSO method, and the compound predissolved in DMSO. Even the very high supersaturated solutions which formed after one particular mixing method were reproducible, demonstrating the effect that mixing has on these systems. There is however no mention of whether the glyburide solid which was obtained after the drying process was crystalline or amorphous – the group do however state that most compounds form a 'dry film' after DMSO removal, which is indicative of an amorphous material.

Typically, no characterisation of the solid produced at the end of kinetic solubility experiments is performed in screening,[37] although there has been a move towards incorporating this into the analysis,[48] and a few characterisation studies have been reported in the literature. Hoelke *et al* analysed the precipitate formed after addition of DMSO stock to aqueous buffer, and found that the precipitate was amorphous in both cases. They suggest that the conditions used in HTS solubility assays, i.e. rapid addition of the compound in DMSO to the buffer, do not promote crystallisation, and that amorphous material is more likely to be formed.[15] The precipitation of amorphous material would be associated with higher solubility values as amorphous materials have a higher solubility than the crystalline solid, although there is not necessarily a large difference between them. Solubility can also vary between polymorphs. Sugano *et al* also explore the nature of the precipitate formed during kinetic solubility experiments using polarised light microscopy (PLM). The group analysed kinetic solubility after a ten minute and 20 hour incubation, and compared these values to the thermodynamic solubility from solid. Characterisation of the solid was performed for both 10 minute and 20 hour incubation times. Many of the drugs precipitated as crystalline, even after only ten minutes. They also found that those compounds which precipitated as amorphous solids showed kinetic experimental solubility values more than five times higher than the solubility from the solid. The final drug concentrations were, however, fairly high in order to allow adequate amounts of solid for analysis.[3]

Other methods have been reported, such as the use of Raman or powder x-ray diffraction for analysis of the precipitate during HTS. Specialised well plates have been developed allowing for direct analysis in the plates. [47,37] However, due to the low concentrations used in HTS, the absolute amount of compound present in a well plate must still pose some issue.

1.3.6 Previous unpublished work in this area

From unpublished work in the Halling lab based on three drug-like compounds, many factors in the sample preparation process influence the amount of solid formed upon mixing of the DMSO and buffer solutions. These variables are not necessarily considered in current experimental set up of either bioassay or kinetic solubility assays, nor is there a huge amount of detailed literature on the subject. These include the way in which the DMSO stock and buffer solutions are added together initially, and the way in which the resulting solutions are subsequently mixed. [49,50] As mentioned, some published work has also demonstrated the effect of shaking and stirring on kinetic solubility results,[37, 38] but no detailed mixing investigation has been performed. The use of sonication post dilution of the DMSO into an aqueous buffer, as recommended in the literature,[29] was also explored, but was found to actually increase the amount of precipitate formed, contradicting the general literature claims.[49] The experiment in the literature[29] investigated the effect of sonication on DMSO stock solutions which were then spiked with 30 % water to induce precipitation, at concentrations of 10 mM – although both DMSO and drug concentration is increased here compared to that used in the work from the Halling lab, the paper does recommend the use of sonication post dilution into aqueous buffer. The results from the literature[29] were that the majority of compounds which had precipitated after dilution of the stock with water were driven back into solution by sonication. The authors did note that 7 compounds showed further precipitation on sonication, and suggested that precipitation was still occurring when the solutions were sonicated, i.e. equilibrium had not been reached. Sonication then increased the speed at which the process was occurring or gave the

system enough energy to overcome nucleation barriers. The paper also states that sonication would only drive the compound back into solution to the extent allowed for by thermodynamics – however due to the input of energy into the system, a stable solid form may be converted to a metastable or amorphous form, which is likely to have a higher solubility than that of the lowest energy solid form, meaning the higher energy form of the compound can then re-dissolve. The samples which did show the ability to be re-dissolved by sonication could have precipitated as e.g. a crystalline solid, which was in equilibrium with the solution, and no further precipitation would occur over time. Then, due to the input of sonic energy, this was transformed to a higher energy form such as an amorphous solid or a higher energy polymorph, which was then not at its equilibrium solubility and so could dissolve into solution. This does suggest that equilibrium is reached quickly for most compounds after dilution of the DSMO stock with water – this contradicts results from the model compound investigated in the Halling lab (erthythrosin), which demonstrated slow precipitation occurring over several days.[49] Sonication would then be expected to increase the amount of solid seen for erthythrosin, as equilibrium behaviour was not demonstrated, in a similar manner to the seven anomalous compounds in the literature. If sonication was seen to drive a particular compound back into solution, the length of time that this effect could last for would depend on the time required for conversion back to the most stable form.

No detailed investigation has been published on the nature of any solid material produced upon mixing DMSO drug stock solutions with buffers. Determination of whether the solid is amorphous or crystalline, and particle size and distribution analyses, would allow further insight into the process which is occurring in these systems. Some limited characterisation has been performed on the nature of the solid formed in kinetic solubility assays (see section 1.3.5). From the previous work carried out in the Halling lab, characterisation of the solid after scale up was performed on two test compounds - one test compound formed an amorphous solid, with another forming crystalline material.[50,51]

Although precipitation is a recognised issue in this area, no detailed work on the factors affecting it has been completed, with only guidelines to minimise its occurrence being seen in the literature. The work from the Halling lab is limited in the number of compounds it has covered, and a wider sample set is need. More detailed investigation into the solid produced is also required in order to fully understand the formation of solid at these low concentrations.

1.4 Aims

The aim of this work is to investigate in detail the factors which influence the precipitation of drug molecules from DMSO and aqueous buffer systems at very low concentrations. Several (typically uncontrolled) variables which may affect the precipitation process are present within these experiments, and these will be explored to ascertain any effect present. These variables include the exact way in which the DMSO stock and aqueous buffer are added together (pipetting variables such as speed, height, and angle of dispension of the liquid), whether the samples are mixed further after initial addition of the organic and aqueous component, the time the DMSO and buffer solutions are incubated for prior to testing, the amount of protein present in the final sample mixture, and the % DMSO present in the final sample. The precipitate formed will be investigated in detail, with regards to the size and number of particles formed, and how these behave kinetically. As there is already published literature on the effects of compound aggregate/particle formation on bioassay results, the work here will investigate the precipitation from a physical chemistry point of view. Performing the work in such a way will allow an understanding of the process itself, as well complementing the already published literature and aiding in interpretation of trends seen in bioassay screening results.

A selection of 3 poorly soluble test compounds will be explored in detail, with the overall aim of rationalising the precipitation for that selection, rather than performing mass screening of tens of compounds. The results will therefore give an indication of the types of behaviour seen, as a starting point for potential further investigation by the industry itself. Both solubility and precipitate analysis will be performed. Various sample preparation methods and techniques for separation of the liquid and solid phases and their effects on the results of kinetic solubility type experiments will be explored. HPLC will be used in determining supernatant and precipitate concentrations. Light scattering, Raman, microscopy and x-ray powder diffraction are some techniques which could be utilised in analysis of the precipitate formed. The form of the precipitate, as well as particle size and growth over time,

are important areas to investigate, and will be linked to both the sample preparation method, and what behaviour is expected based on crystallisation theory at higher concentrations, and the literature available. This work has practical relevance to the pharmaceutical industry, as well as the novelty of exploring precipitation and/or crystallisation of organic compounds at these low concentrations.

The main difficulty in this work is the low concentration of drug (in the micromolar concentration region) present in the samples – this makes any precipitation difficult to see, and any material formed difficult to analyse.

As there is little published experimental data of the type performed here, where an effect was seen, the results were written up as a series of research articles with a view for publication. Since various different aspects were explored which were suited to be published separately, the results are presented in this thesis as a compilation of research papers (chapter 2 onwards). One article is already published (chapter 2).[52] The author of this thesis performed all of the experimental work detailed, and undertook the preparation of the majority of the first draft of every paper. Subsequent corrections and addition of comments to the draft papers from the other contributing authors were also performed by the author of this thesis.

1.5 Model Compound Selection

1.5.1 Compilation of initial compound list

Model compounds were required to meet four basic, practically relevant criteria;

1. The compounds must be poorly water soluble, as only low solubility compounds are likely to precipitate at the low concentrations relevant to screening.
2. The compounds should be commercially available, and known to be biologically active. This latter criterion gives the results relevance to the pharmaceutical industry.
3. Following on from 2, whilst it is desirable for the compound to be a known pharmaceutical, it should not be too toxic. This criterion was included for safety purposes.
4. The compounds should have a UV absorbance. This allows straightforward analysis.

In discussion with MSD, a shortlist of eleven commercially available compounds was compiled. All compounds met the criteria set out above. The compounds were judged to have low solubility based on results of in-house kinetic solubility experiments and those available online at the Physchem forum website.[53] The original eleven compounds were as follows;

Table I: 11 compounds on which preliminary experiments were performed

Compound Name	MW (g/mol)	Product No.	Lot No.	Storage Requirements
Amiodarone HCl	681.8	Sigma A-8423	87H0597	2-8°C
Clotrimazole	344.8	Sigma C-6019	118H1359	Room Temp
Thioridazine HCl	407.0	Sigma T-9025	87H0803	Room Temp
Tolnaftate	307.4	Sigma T-6638	43H0109	2-8°C
Imipramine HCl	316.9	Sigma I-7379	48H0362	Light Sensitive. Room Temp.
Hydrocortisone 21-Acetate	404.5	Sigma H-4126	78H0468	Light Sensitive. Room Temp.
Promethazine HCl	320.9	Sigma P-4651	128H1474	Light Sensitive. Room Temp.
Bumetanide	364.4	Sigma B3023	021K1134	No special storage requirements
Carbamazepine	236.3	Sigma C-4024	121K1510	Light Sensitive. 2-8°C
Metroprolol Tartrate	684.8	Sigma M-5391	101K1517	Light Sensitive. Room Temp.
Tolbutamide	270.3	Sigma T-0891	061K1629	Room Temp

All eleven compounds were subjected to several scoping precipitation experiments, with the intention of having a first look at how the compounds behaved under conditions relevant to screening. The results from these experiments were then used to narrow the list to only those compounds which showed precipitation and were stable under the conditions investigated. The eight excluded compounds and the reasoning behind this exclusion are as follows –

- Four compounds (carbamazepine, tolbutamide, metroprolol tartrate and hydrocortisone acetate) showed no precipitation under the conditions trialled here. Variables investigated include initial drug concentration (up to 500 μM), pH and incubation time. None of these four compounds demonstrated any tendency to precipitate during the course of the scoping experiments. They were therefore disregarded.
- Two compounds (imipramine HCl and promethazine HCl) demonstrated decreasing recoveries over short time periods, suggesting a tendency to decompose rapidly in the buffer used. Due to this possible degradation, no in depth experiments were performed with these compounds.
- Thioridazine HCl and bumetanide did not show any of the undesirable characteristics mentioned above. The reason for their exclusion in further experiments was merely due to the fact that more data had been collected on some of the remaining compounds during the initial trials, and it made more sense to continue on with those.

This streamlining process left three compounds (amiodarone HCl , clotrimazole and tolnaftate) for detailed investigations.

1.5.2 Model compounds used in detailed experiments – general information

Amiodarone HCl is the salt of a weak base, and has use as an antiarrythmic agent. The pKa of the tertiary amine has been reported as 10.2[54] and 8.73 ± 0.05.[55]

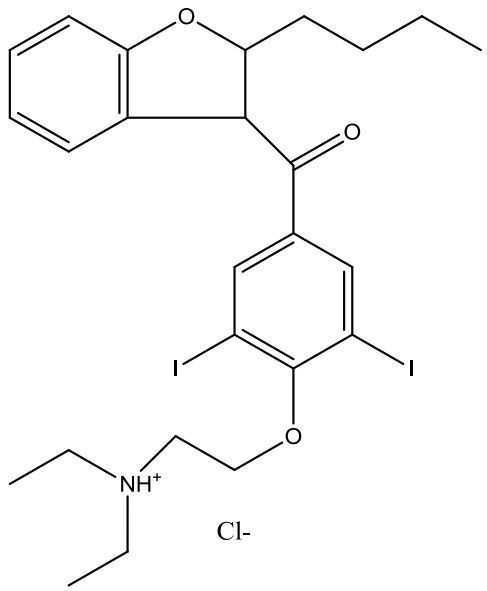

Figure 2: Amiodarone HCl structure

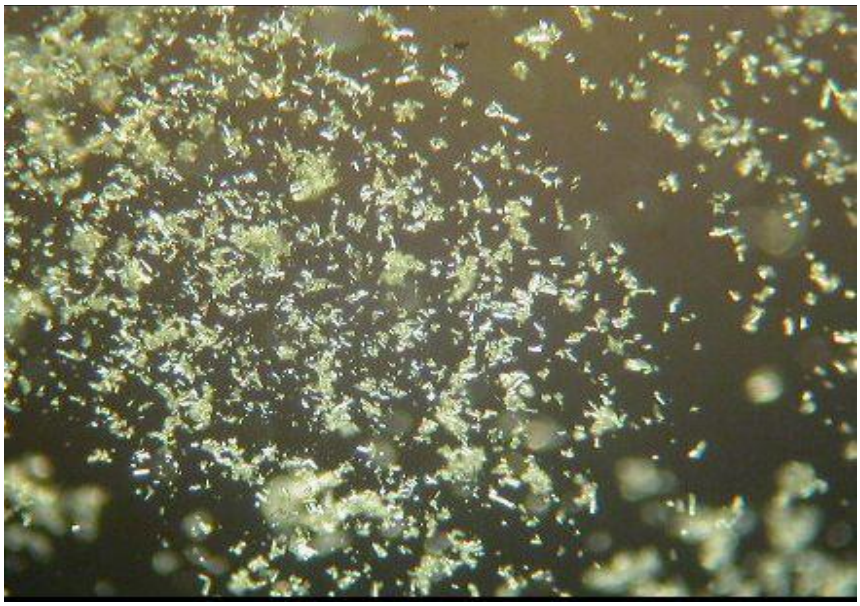

Figure 3: Amiodarone HCl starting material, viewed under a microscope with a polarising filter

Clotrimazole is a weak base, with a pKa of 6.1.[56] The drug has anti fungal applications, and is typically used commercially as a topical treatment.[57]

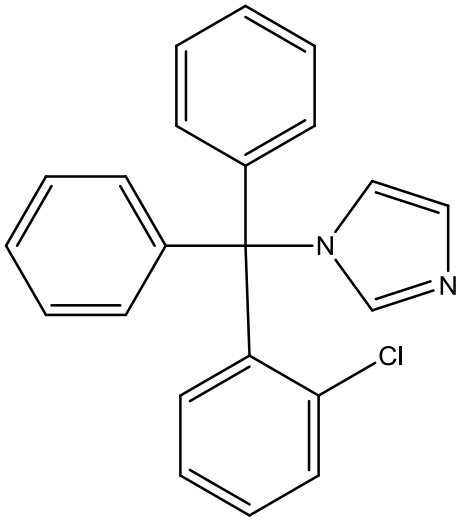

Figure 4: Clotrimazole structure

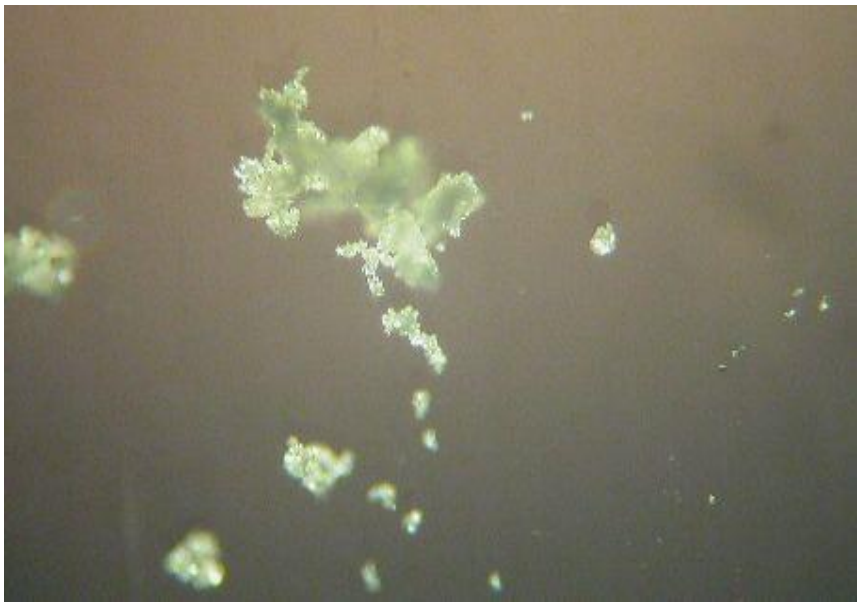

Figure 5: Clotrimazole starting material, viewed under a microscope with a polarising filter

Tolnaftate is a commercially available, neutral compound. It is a squalene epoxidase inhibitor and also has applications as an anti-fungal agent, typically as an athlete's foot treatment.

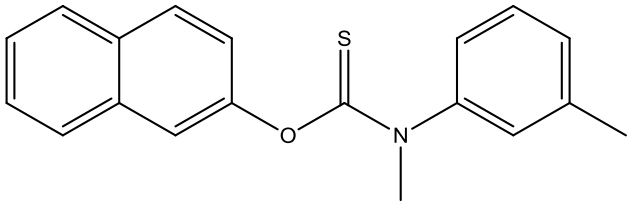

Figure 6: Tolnaftate Structure

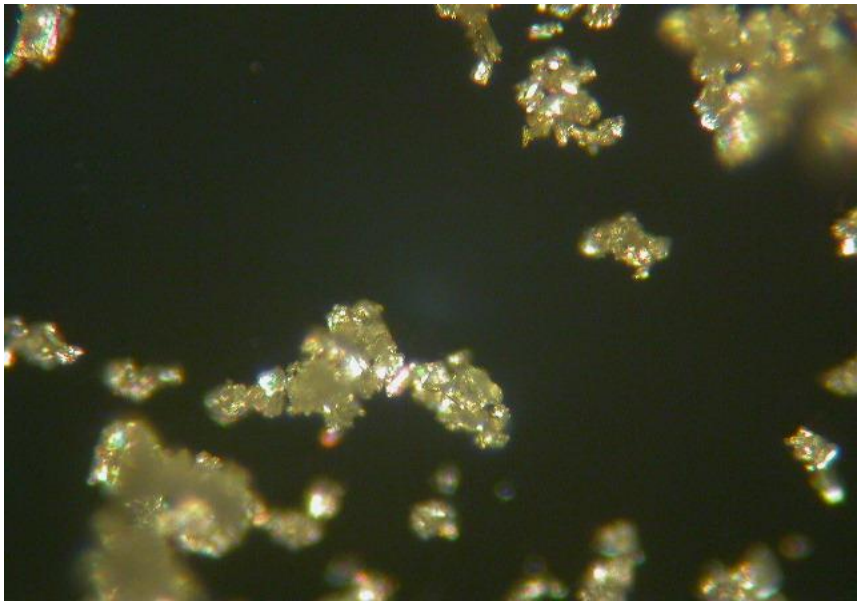

Figure 7: Tolnaftate starting material, viewed under a microscope with a polarising filter

All starting materials used in the studies described were purchased from Sigma Aldrich, and used without further purification. NMR was used to confirm compound purity, and all three compounds either matched or exceeded manufacturer's specifications (≥ 98%). NMR spectra are available in the appendix. XRPD spectra of all starting material were also obtained, for use as reference spectra for any XRPD analysis. These are also available in the appendix.

Table II: Detailed compound information

Compound	*Merck Index Solubility Description*	*Literature Solubility Range*	*Polymorphism seen previously?*
Amiodarone HCl	'0.07 g/100 mL water' (~**1 mM**)	**3 µM, < 2 µM**, pH 7.4[53] ~**0.2 mM** (<0.125 mg/mL) H_2O, 20 °C[58] **~1 mM** (0.716 g/litre) H_2O, 25 °C[59]	None found documented in literature.
Clotrimazole	'practically insoluble in water'	**1.45** µM (0.5 µg/mL), 'aqueous'[56] **5.5 µM**, 'aqueous' [60] **15 µM**,[53] **0 µM**[53] and **3 µM**, pH 7.4[53]	Yes – but only from melt. [61]
Tolnaftate	'practically insoluble in water'	**<3** and **3 µM**, pH 7.4[53]	None found documented in literature.

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2. Monitoring of particle growth at a low concentration of a poorly water soluble drug using the NanoSight LM20

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(Published in; Colloids and Surfaces A: Physicochemical and Engineering Aspects, 384, 1-3, p. 233-239)

2.1 Abstract

The purpose of this work was to investigate the precipitation of a poorly water soluble drug (tolnaftate) from low, µM concentration solutions. This was to test the applicability of nanoparticle tracking analysis (NTA; the NanoSight instrument), with comparison to results from dynamic light scattering (DLS).

Samples containing 30 µM of tolnaftate, 1% DMSO (by volume) were prepared by mixing a concentrated DMSO stock solution and an aqueous buffer. The samples were then analysed over time either using the NanoSight instrument or by DLS. Obtaining meaningful results from the former required careful attention to instrument settings.

From NTA there was initially a fairly narrow size distribution around 200 nm, with a concentration of around $4x10^8$ nanoparticles/mL. Over 3 hours, the particles grew,

with increasing polydispersity, and a skewed distribution up to 800 nm, while the concentration fell to around 1x 10^8 particles/mL. DLS was consistent in showing the size increase, but could not detect the remaining smaller particles and polydispersity.

Conclusions: The growth of particles of a poorly water soluble drug was successfully monitored using NTA, which gives additional information not offered by DLS. Nanoparticle precipitation at the concentrations used here is of relevance to high throughput screening in early drug discovery.

Keywords; HTS; solubility; nanoparticle growth; NTA; DLS; Tolnaftate

2.2 Introduction

The growth of organic and inorganic crystals has been studied extensively using a variety of techniques.[1-7] This work has been motivated in relation to preparative crystallisation, and hence has used high solute concentrations, typically 0.1 to 1 M. In comparison, almost nothing is known about growth of crystals or other precipitate particles from very low dissolved concentrations. Precipitation or crystallisation of poorly soluble organics from μM concentrations is however of practical importance, for example in studies of candidate pharmaceuticals. [8] Solutions in dimethylsulfoxide (DMSO) are added to aqueous media during testing for biological activity [9,10] or solubility.[11,12] In the latter case precipitation is expected, while in the former it may occur.[9] We now demonstrate how formation and growth of precipitate particles in such low concentration systems can be monitored using a novel optical technique that detects and characterises individual particles.

The technique of nanoparticle tracking analysis is a relatively new approach to particle size and concentration measurement, implemented in the commercial NanoSight LM20 instrument. Recent papers have looked at several different applications of the instrument.[13-16] The sample is introduced into a viewing unit, and an image of the particles' scattering of the laser light is captured by a CCD camera, set on a microscope. A video of the sample is recorded, and then processed. Each individual particle seen is 'tracked' by the nanoparticle tracking analysis (NTA) software. From the Brownian motion, particle diameter can be calculated using the Stokes-Einstein equation.[17] The displacement co-efficient (Dt) from the mean squared displacement of the particle tracked is calculated, and substituting this value into the Stokes-Einstein equation allows calculation of particle size. Estimation of the concentration of the particles is based on the assumed scattering volume calculated from the dimensions of the field of view (at a given magnification) and the depth of the laser beam. The average number of particles per millilitre of sample is then simply extrapolated from the assumed scattering volume. The instrument differs to other light scattering techniques such as dynamic light

scattering (DLS) in that an image of the particles' scattering (although not the particle itself) is recorded. The concentration of particles present and their size distribution can be estimated based on the tracking of individual particles, unlike DLS.[18] In contrast, in DLS the signal is dominated by the larger, more intensely scattering particles, and the result is an intensity weighted mean size. It is also very difficult to obtain information on the concentration of particles present in the sample from DLS. The two techniques are complementary to one another, and both offer their own advantages and disadvantages, as discussed extensively by Filipe et al.[17] In the work done here, the NanoSight LM20 and NTA 2.0 software were used to measure the growth of particles of a poorly soluble drug compound, tolnaftate (Tol), over time. The sample concentration of around 30 μM is within the range of that used in pharmaceutical screening, and the data here provides an insight into how poorly soluble drug molecules behave at low concentrations upon addition of DMSO stock to an aqueous system. The effects of instrument settings were investigated in detail, and sensible choices suggested. The results from the NanoSight were then compared to those obtained by DLS.

2.3 Materials and Methods

2.3.1 Tolnaftate Sample Preparation

Tolnaftate (T6638) was purchased from Sigma Aldrich, and the purity was confirmed by ^{1}H-NMR (>99%).

A 0.05 M phosphate buffer solution was prepared, using 0.025 moles of KH_2PO_4 and 0.025 moles of $Na_2HPO_4.2H_2O$ per litre of deionised (Millipore, Direct Q, ultrafiltered) water. The measured pH was 7.0 ±0.1. The buffer was then filtered using 0.2 μm polyethersulfone (Sartorius-Stedim, Vivaspin PES 0.2 μm) centrifuge filters (3 mins, 1037 x g RCF) to remove any particles present.

The DMSO used was SAFC Methyl sulfoxide, >99%.

A 2.99 mM stock solution of the drug was prepared in DMSO. A volume (10 μL) of this stock solution was pipetted into a 1.5 mL glass HPLC vial. The filtered buffer (990 μL) was then added to the HPLC vial containing the tolnaftate solution. This gave a solution at a concentration of 29.9 μM tolnaftate, with a 1% DMSO concentration. Each sample was inverted by hand approximately ten times before introduction into the NanoSight sample chamber or the cuvette used in DLS.

2.3.2 NanoSight – Sample Procedure

After introduction into the NanoSight sample chamber, the sample was left to equilibrate to the temperature of the chamber for 30 seconds (as recommended in the NanoSight LM20 and NTA 2.0 software manual. Software version used – release version build 0132). The first video (t=0) was captured after this equilibration time, with subsequent videos captured at *x* minutes after t=0. The instrument used did not have the capacity to control temperature, and so each time course was performed at room temperature. At time 0, the average temperature recorded from the three replicates was 21.8 ± 2.4 °C, and at t=180 minutes the average recorded temperature had risen to 23.7 ± 2.0 °C. The outputs from the NTA

software were strongly dependent on the instrument settings used, and the next sections explain in detail our observations here and the rationale of choices made.

2.3.3 Video Capture Settings

The camera settings for the instrument were set using the 'Autosettings' option on the software – this allows the software to optimise the shutter and gain settings and was thought to give less chance of bias than optimising manually for every sample. The shutter setting applied is stated in the NanoSight LM20 and NTA 2.0 software operating manual as affecting the length of time the camera shutter is open for each frame, therefore controlling the amount of light captured from the particles. The gain setting controls the sensitivity of the camera.

The focus used was judged by eye, and was adjusted so that the majority of particles seen were in focus at any one time. A test of repeated de-focusing and re-focusing on a sample no longer changing significantly with time showed that results were reproducible. This reproducibility was demonstrated by performing seven measurements of the same sample (a suspension of thioridazine HCl). The results of this were an average diameter of 251 $\pm$ 22 nm, and an average concentration of $0.7x10^8 \pm 0.2x10^8$ particles/mL. This experiment therefore validated the focusing used in the time course experiments. The position in the X and Y planes was chosen in accordance with the description of the optimum viewing position in the NanoSight manual. First, the intense patch of light corresponding to that of the laser was located. The field of view was moved slightly to the right, and the Z-plane adjusted to focus until the light to the right of the laser patch converged into a line. The position was then moved one field of view to the right, so that the laser line was not in view.[18] The X-Y viewing position was not changed during the time course.

The accuracy of particle size distribution data is expected to increase with the total number of particles whose size is determined. Hence each video was recorded for the maximum time allowed for by the instrument (215 seconds).

2.3.4 Processing Parameters

The processing parameters of brightness and gain were not changed due to the video contrast already being optimised by the programme.

A blur (smoothing setting) of 5x5 was employed, following the recommendation in the operating manual,[18] which states that if automatic threshold detection was employed (which it was for all samples), the blur setting should be increased by one level than that normally used. The threshold detection value determines the minimum grey scale value of any 'blob' image necessary for it to qualify as a particle to be tracked.

The minimum expected particle size was set at 100 nm for all samples. Reprocessing the data with the lowest minimum particle size (30 nm) did not significantly change the results. The minimum track length was set to automatic, with the software calculating this based on the particles in the video. The minimum track length setting defines the minimum number of steps a particle must take before its size value can be accepted for inclusion in the particle size distribution plot. Figure 1 shows a snapshot of the software processing the video, and tracking the particles. The red and blue lines represent the particles' trajectory under Brownian motion, with the lines which have turned red representing those particles which have been tracked for a long enough duration to have their size included in the size distribution data. The smoothed particle size distribution curve can also be seen – the distribution changes dynamically as more particles are tracked and added to the plot.

Reprocessing the same video would not give consistent results due to a bug present in the software (shutter and gain settings were taken from the current instrument state, rather than those recorded when the video was acquired). Therefore, if the video was not processed immediately after recording, the shutter and gain were set manually to those used in the capture of the video.

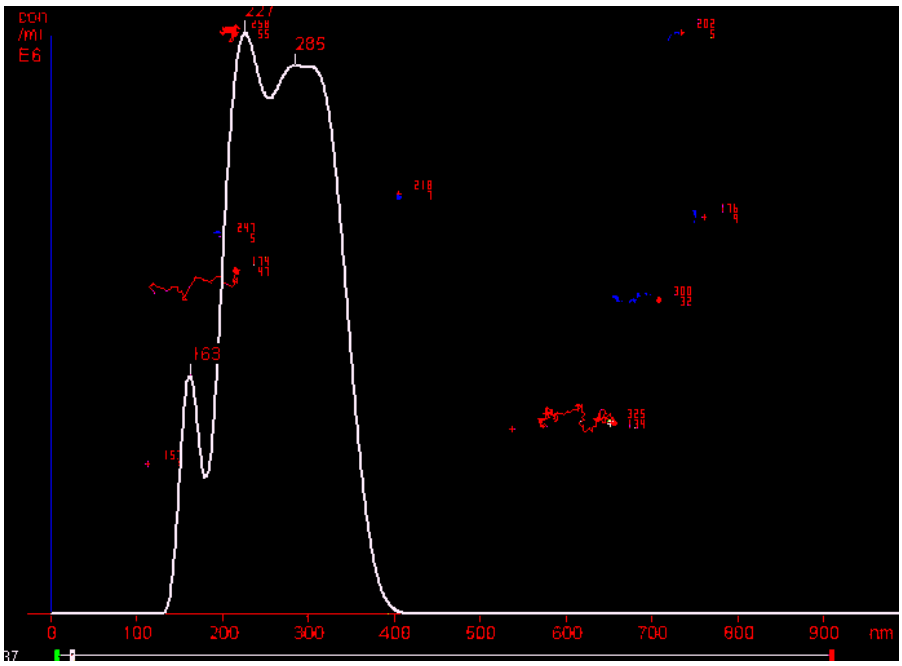

Figure 1: Still of software 'tracking' particles

The data obtained from the NanoSight was adjusted for e.g. drift and camera settings by the instrument. These calculations occur 'behind the scenes' in the software and no file is available which details the corrections made. After adjustment, the data are then smoothed by the instrument using a moving average, to give a plot similar to that seen in figure 1. In order to obtain data which were adjusted as appropriate by the software, but which was not smoothed, 'Ctrl + 0' was pressed prior to processing each video – this adjusted, but not smoothed, data are that which will be discussed further with relation to the NanoSight. The mean obtained from the NanoSight instrument is a number weighted average. From the distribution obtained from the NanoSight output, volume and intensity weighted means could be calculated for each time point.

2.3.5 NanoSight – Preparation and analysis of a blank sample

A blank sample was prepared by pipetting 10 µL of DMSO into an HPLC vial. 990 µL of filtered pH 7 phosphate buffer was added, and the sample inverted approximately ten times by hand. The blank was then loaded into the sample chamber, and left to equilibrate for 30 seconds. The camera autosettings option

was used to locate any small particles in the blank, and these were then brought into focus. The camera shutter and gain settings were then changed, first to the higher, and then to the lower, limits of these setting which were used in recording the actual samples.

2.3.6 DLS Procedure – Sample

The filtered phosphate buffer was used to prepare a 1% DMSO/99% buffer blank. Analysis of this solution by the DLS showed that some particles were present, as the autocorrelation function was not completely flat. The buffer was then filtered again using a 0.2 μm PTFE hydrophilic (Millipore) syringe filter. These double filtered blanks also showed some particles in solution. The buffer which had been double filtered was used to prepare the sample.

The DLS instrument used was a Malvern High Performance Particle Sizer, (HPPS 3.3), with non-invasive back scatter (NIBS) technology. The scattering angle was 173 °. The laser wavelength used was 632 nm. A 20 nm calibration standard was checked using the DLS, with the size given as 19.5 nm, demonstrating that the instrument was working accurately.

The DLS instrument was programmed to make one measurement every ten minutes, with the first measurement being t=0, and to perform 5x20 sec acquisitions on each sample, with the average result quoted for that time point. The instrument automatically optimised the position of the laser and the attenuation depending on the sample. The instrument was set to 25°C for all experiments. The refractive index used for tolnaftate was 1.697, the value for the crystalline solid.[19]

2.3.7 Preparation and analysis of a blank – DLS

As described in section 2.3.6, the buffer was filtered, then filtered again. A 1% DMSO/buffer solution was prepared using the double filtered buffer, and analysed using the Malvern instrument.

2.4 Results and Discussion

2.4.1 NanoSight Results

To obtain meaningful results it was necessary to pay careful attention to a number of instrument settings that would significantly affect the outputs, as explained in detail in section 2.2 above. The blank samples showed no particles visible at the camera settings used, eliminating this as a source of any particles seen in the samples.

In total, three time course experiments at the same sample concentration were performed using the NanoSight. The results from all three showed similar trends over time - particle size increased, distribution changed from relatively monodisperse to polydisperse, and particle concentration decreased. Whilst different particle diameters and concentrations were obtained for the three experiments, the overall trends were similar and for a nucleation dependent process such as this, variability between samples is expected. The results from one of the tolnaftate experiments are shown in table I and discussed in more detail. The data for the remaining two time course experiments is present in the appendix.

Table I: Mean diameter and concentration with time obtained from NTA

Time (mins)	Number Mean (nm)	Standard Deviation (nm)	Concentration (10^8 particles per mL)	No. of Completed Tracks
0	206	49	4.41	1516
10	240	54	1.92	642
20	251	56	1.57	444
30	289	71	1.99	505
60	291	69	1.72	425
90	299	66	1.33	300
120	326	90	0.76	166
150	373	125	1.27	257
180	378	138	1.52	293

The standard deviation in column three is a measure of the spread of particle sizes in the particle size distribution, and is not an indication of error in the mean.

The mean and standard deviation shown have been calculated from the raw data, rather than the smoothed NanoSight output. The concentration and number of completed tracks have been taken from the NanoSight output files. Each completed track corresponds to a particle whose size has been included in the distribution. Stills from the first frame of the videos for t=0 and t=180 minutes are shown in figures 2 and 3.

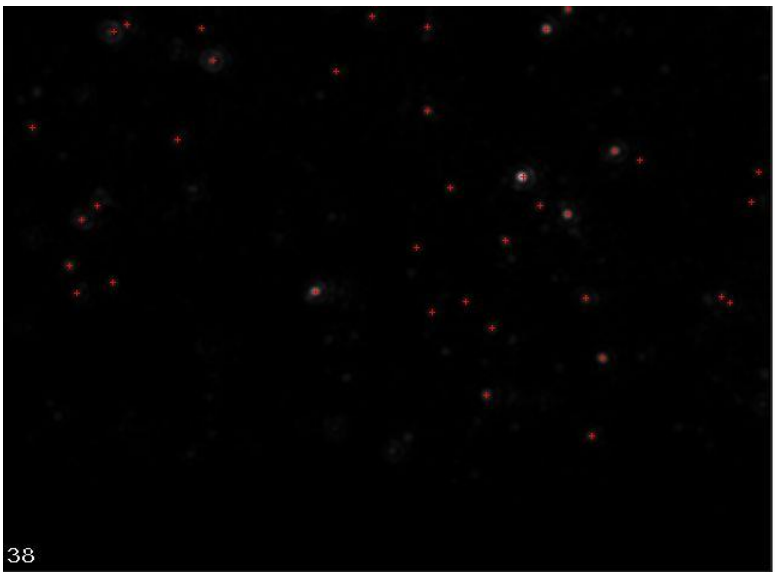

Figure 2: Still of video from Tolnaftate 29.9 µM at t=0 minutes (38 visible particles)

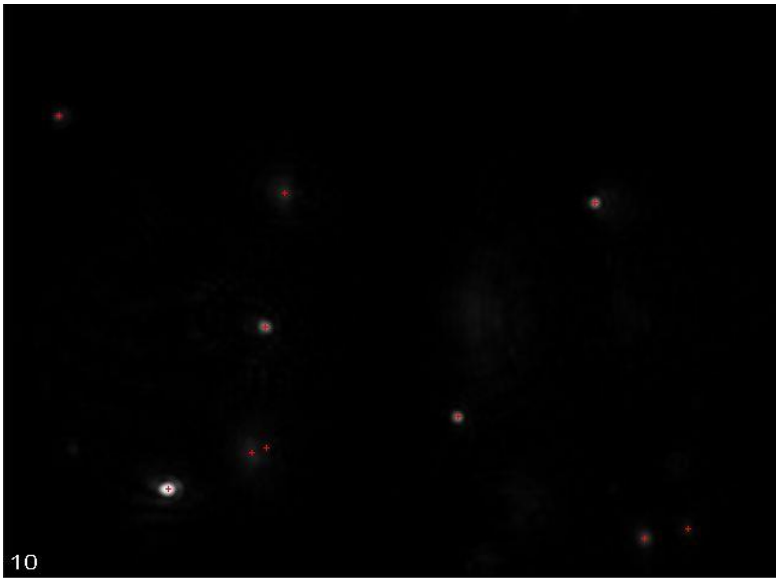

Figure 3: Still from video of Tolnaftate 29.9 µM at t=180 minutes (10 visible particles)

At t=0, there is a large number of particles visible (38) with the NanoSight, which is what would be expected due to the low solubility of the drug. It is not, however, obvious from the solubility that the particles present are nanoparticles within the size range which can be analysed by the NanoSight. Seidler et al have demonstrated that some small organic molecules with poor solubility can form aggregates of nanometre size in aqueous solution, which inhibit enzymes promiscuously during screening, and have measured the size of these aggregates using DLS.[20] Here, the

NanoSight is used to monitor both the size of the particles formed and how they change over time, and the results compared to DLS. No enzyme inhibition studies were performed. The image of the same sample at t=180 minutes shows that there are fewer particles. The red crosses shown on the images represent the particles that the software 'sees' and will track. The number at the bottom left corner of the image is the total number of particles the software can 'see' in this particular frame.

Although for the three replicate experiments performed the actual particle sizes varied, all three demonstrated the same trend of increasing size over time. The standard deviation increases with each time point, demonstrating the increasing spread of data as the system becomes more polydisperse, as shown in table 1. This can also be seen from the overlay of distributions at different time points (figure 4).

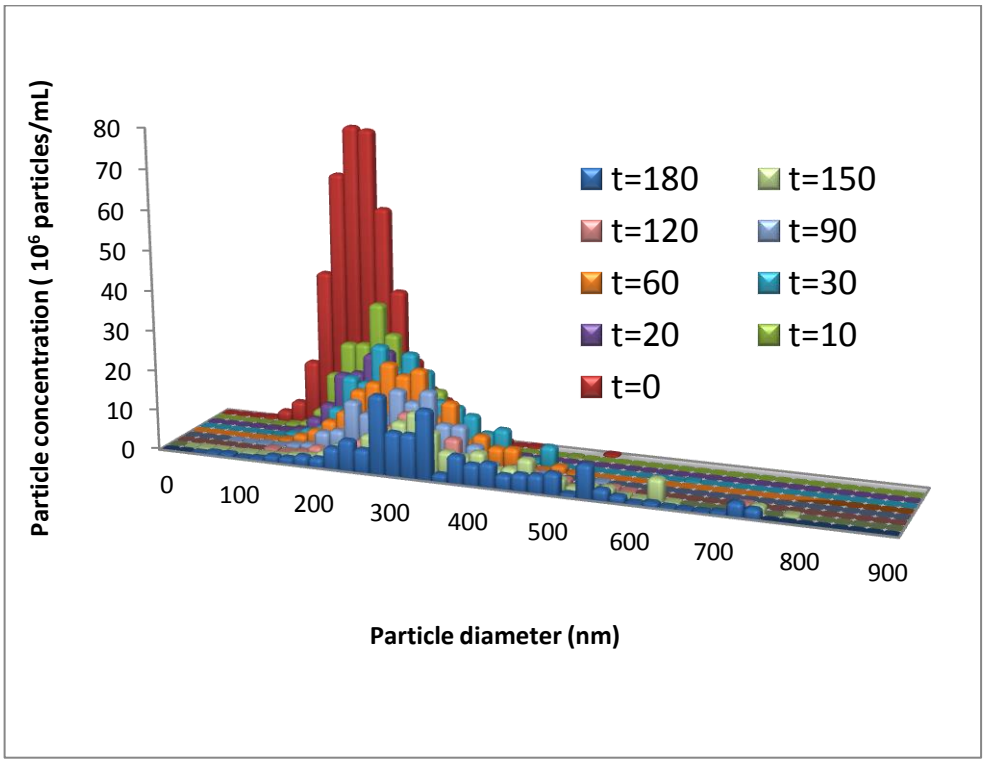

Figure 4: Overlay of NTA particle size distributions obtained from tolnaftate samples at various time points

The distribution at t=0 has a sharp monodisperse profile. As time increases, the sample profiles gradually become more polydisperse and ill-defined.

The mean particle diameter was plotted as a function of time, resulting in the graph shown in Figure 5. Mean particle diameter shows a clear upward trend over the three hour time period.

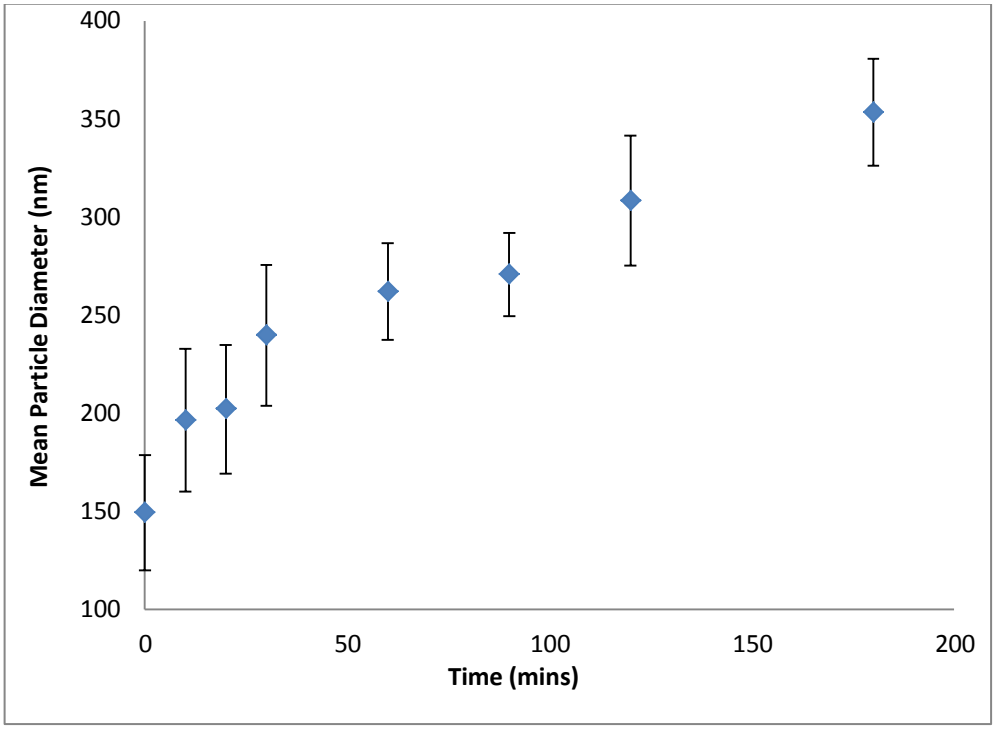

Figure 5: Mean particle diameter versus time obtained from the NanoSight instrument – error bars represent standard deviations (n=3)

Figure 5 shows the average diameter across the three repetitions performed at each time point, plus or minus the standard deviation in the mean.

It can be seen from figure 4 that as well as mean particle size increasing, there are populations of larger particles emerging over time. It is also possible to see that, after an initial fall, the concentration of the smallest particles also increases again at longer times: this was clearly seen in the raw data. This is consistent with Ostwald ripening, where smaller particles become smaller, and larger particles become larger. Ostwald ripening suggests that particle size distribution should tend to become more uniform over time as the particles tend to larger sizes.[3] Whilst this is not observed in these samples, this could be because the latest time point here is not the end point of the process, and if the sample were left for long enough, the

distribution would re-align towards that of a monodisperse population. It could also be the case that at the initial time point, there are particles present which are too small to be detected by the NanoSight. These would re-dissolve into the bulk solution, and then precipitate back onto the larger particles, which is more favourable. This is, however, speculative. If the particles were truly monodisperse, little ripening would occur. If growth were the only mechanism responsible for the trend seen, there would be no emergence of smaller particles over time. Another possibility is that more drug precipitates over time, and due to the reduction in super saturation, initial particle size is reduced. Since no information on the crystallinity of the particles is obtained from the NanoSight, theoretically the initial particles could be amorphous – over time they could re-dissolve, and then undergo conversion to a more stable crystalline form, and this is what is present in the small particle populations at later time points.[3] One other possibility may be that a metastable polymorph precipitates, then undergoes a solution mediated phase transformation to a less soluble form, which then grows.[21] There is also a possible contribution from the artefact of particles settling out of the measurement volume or becoming stuck to surfaces over time. From the data obtained here it is impossible to know which of these is occurring, or whether several are in fact occurring at once.

The mass of drug present in the particles per volume unit can be estimated by calculating the volume fraction occupied by the particles, then multiplying by the density of the solid (took to be 1.223 g/cm^3 for tolnaftate, that of the crystalline material).[19] It should be noted that for this calculation the particles are assumed to be solid, non porous spheres. This mass concentration can then be converted into a molar concentration using the molecular weight of tolnaftate (307.4 g/mol). The amount of drug present in the particles as a percent of the initial added mass (29.9 nanomoles) can also be calculated.

$$\boldsymbol{Volume\ fraction} = \frac{\boldsymbol{\pi}}{\boldsymbol{6}}\boldsymbol{d^3}(\boldsymbol{cm}) \times \boldsymbol{concentration}\ (\boldsymbol{no.particles/mL})$$

Equation 1

$$\boldsymbol{Mass\ of\ drug\ in\ particles\ (g\ per\ mL) = Volume\ fraction \times \rho}$$

Equation 2

Table II: Calculated number of nanomoles of drug present at each time interval based on NanoSight results from one measurement, calculated using equations 1 and 2

Time (mins)	Volume mean (adjusted not smoothed) (nm)	Concentration (10^8 particles per mL)	No. of nanomoles of tolnaftate present in the particles	% of drug added
0	218	4.41	9.51	32
10	253	1.92	6.47	22
20	264	1.57	6.01	20
30	307	1.99	11.99	40
60	308	1.72	10.46	35
90	314	1.33	8.57	29
120	353	0.76	6.96	23
150	415	1.27	18.90	63
180	428	1.52	24.81	83

Overall, an increase in the number of nanomoles of drug present in the particles is seen, in particular for the last two time points, suggesting that further precipitation is occurring as well as growth and ripening of the particles already present. The random nature of the results prior to the last two time points reflect the errors within the concentration measurement. Since an increase is seen, it is likely that further precipitation is occurring, rather than just ripening of the particles already present. By the end of the three hour time period, the results from the NanoSight suggest that around 83% of the drug added is present in the form of nanoparticles.

Full details of the calculation used here is seen the appendix (chapter 2 additional data).

A decrease in the number of particles per mL (from the NanoSight output) over time is also seen (figure 6), which would be expected due to particle growth. The concentration does not drop steeply however, again giving support to the theory that several processes are occurring at once, as discussed above.

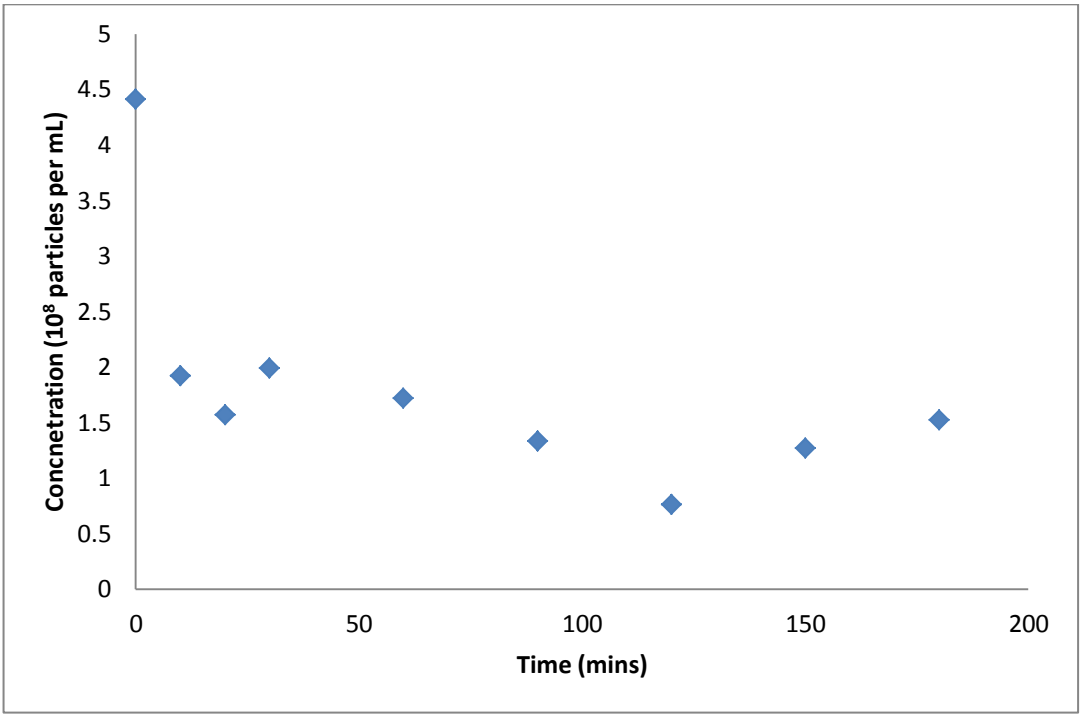

Figure 6: Particle Concentration Vs Time

2.4.2 DLS results

Some particles were still present in the blank sample even after the double filtration, however the autocorrelation function (the standard output of a DLS) for the blanks and the samples are completely different, eliminating the particles in the blank as a cause of the data recorded for the samples. This is shown in figure 7.

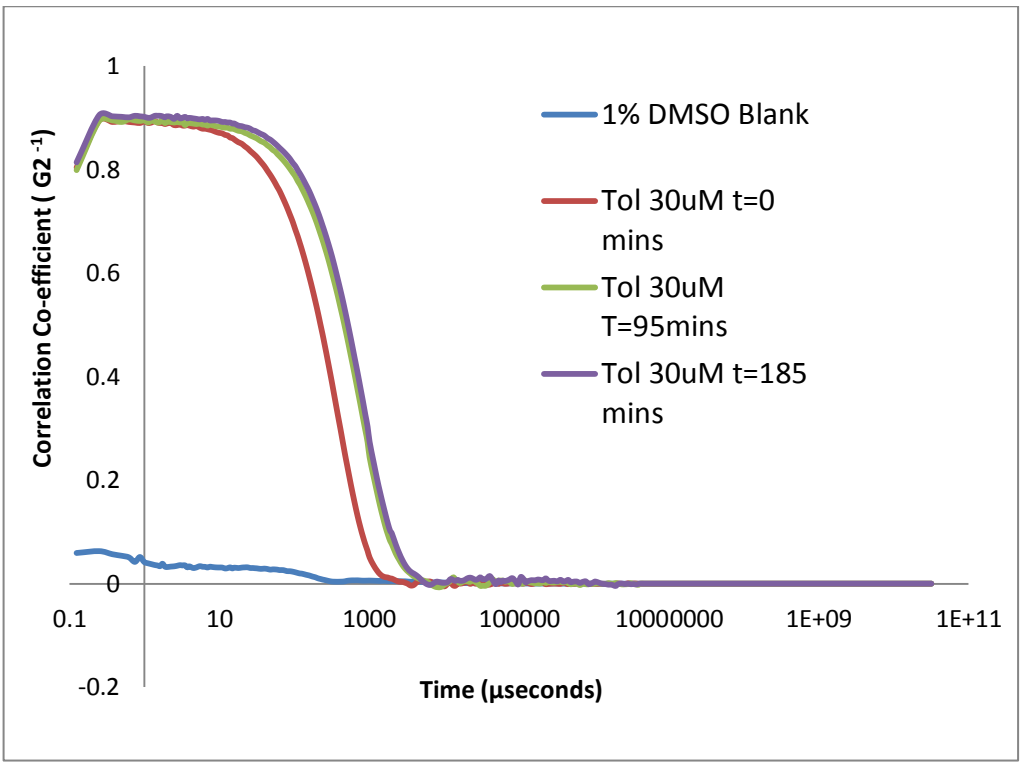

Figure 7: Autocorrelation functions

The blue line at the bottom of the chart is the autocorrelation function relating to the blank, with the other lines being the autocorrelation functions at different time points for one 29.9 µM sample.

As with the NanoSight, three replicate analyses were performed, and each time, the Z-averaged (intensity weighted) hydrodynamic diameter increased over the course of 3 hours. A decrease in mean count rate was seen for each sample. Differences were seen in the mean sizes between replicates, however this is thought to be due to the sample itself rather than the technique used to measure the particles as discussed previously. As with the NanoSight, the results from one of the samples are shown in more detail in table III.

Table III: DLS Results, Z average diameter, count rate and PDI

Time (minutes)	Z average hydrodynamic diameter (nm)	Count rate (kcps)	Attenuator	Position (mm)	Polydispersity index (PDI)
0	269	453.4	8	2	0.144
12	345	448.7	8	2	0.019
25	411	449.1	8	2	0.118
37	460	451	8	2	0.096
62	475	432.9	8	2	0.078
99	545	419.6	8	2	0.036
123	558	400.3	8	2	0.042
157	543	375.6	8	2	0.126
185	603	367	8	2	0.053

A plot of hydrodynamic diameter versus time shows an upward trend, similar to that seen for the NanoSight samples (figure 8).

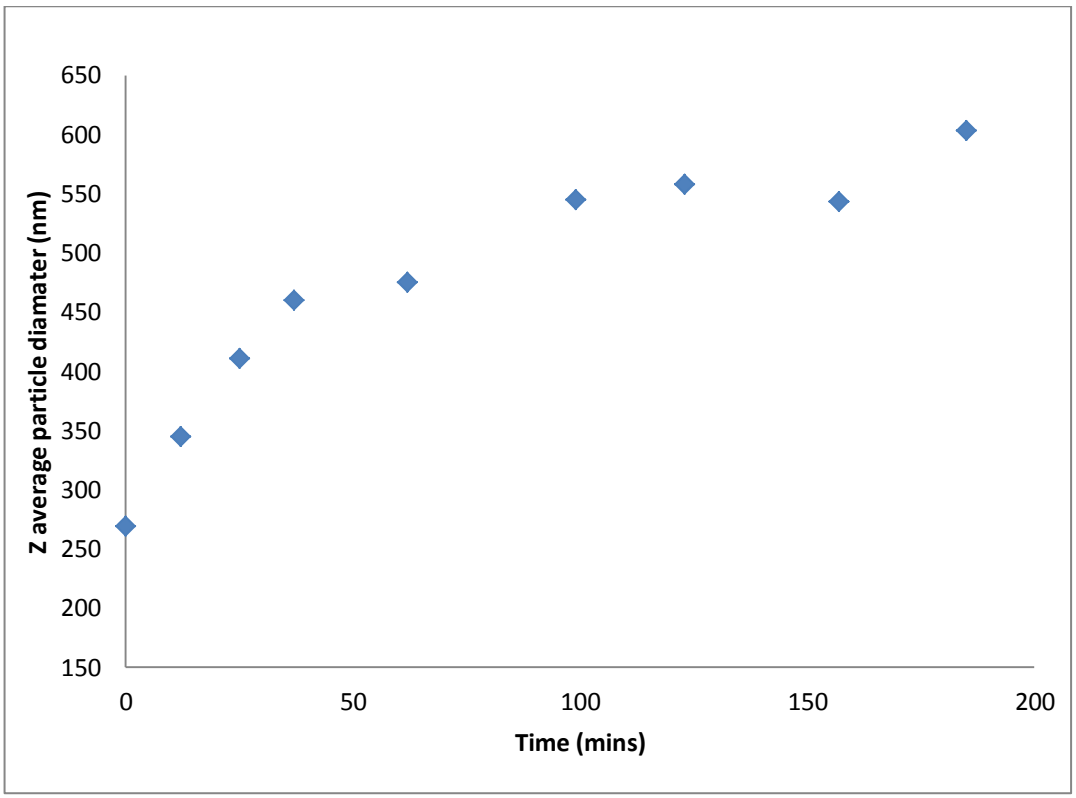

Figure 8: Mean particle diameter versus time, DLS

Although the mean particle diameters obtained from DLS are intensity weighted averages, and those obtained from the Nanosight are number weighted averages, the trend seen is similar. Since the attenuator and measurement position was the same for all time points, the count rate can be compared between measurements. The count rate is a measure of the scattering intensity displayed by the sample. Since larger particles scatter more intensely, it would be expected that the count rate increase with time, however this is not observed. The main difference in the data obtained from the NanoSight and that obtained from DLS is that the DLS results do not show the change from monodisperse to polydisperse with time. The polydispersity index (PDI) shows only random variation with time, and even at the end time point is still very low. Filipe et al[17] found that DLS cannot resolve polydisperse samples, whilst the NanoSight can - this is in line with the results shown in the present work.

In order to compare the average particles sizes obtained from the NanoSight to those from the DLS, an intensity weighted mean was calculated from the particle distributions obtained from the NanoSight for each time point. Since DLS is an intensity weighted mean, there is a bias in the mean towards larger, more intensely scattering particles, with low concentrations of larger particles being easily detected, and affecting the measurement. Filipe et al analysed a mixture of 100, 400 and 1000 nm polystyrene bead using DLS – the results showed a monodisperse sample of 700 nm, due to the masking of the smaller by the larger particles.[17] The NanoSight was however able to resolve the three particle size populations – similar results are seen here. The average from the DLS is likely to be biased towards the larger particles, both due to the weighting of intensity, and possibly the masking of the smaller particles in the sample by the larger ones. The DLS mean would therefore still be expected to be larger than that of the calculated NanoSight intensity mean. In order to represent the particles measured by the DLS, the diameter of the 95th percentile of each NanoSight time point was calculated and plotted in figure 9, along with the results from the DLS, the NanoSight number mean and the NanoSight calculated intensity mean.

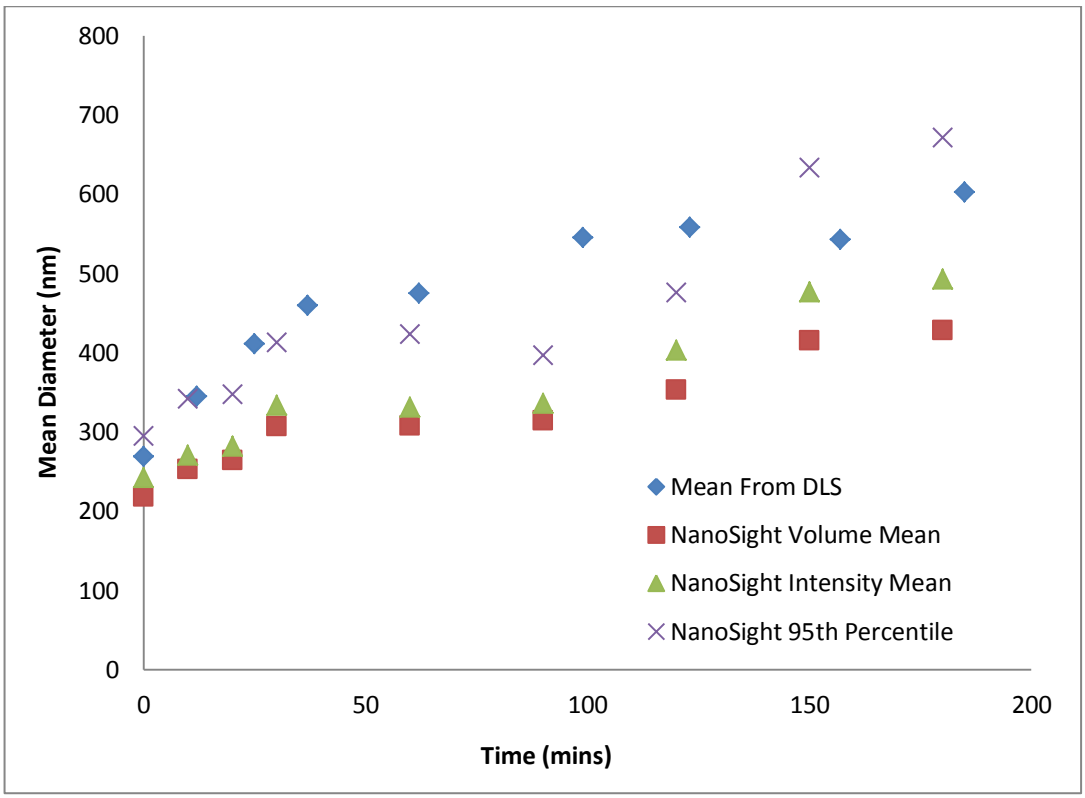

Figure 9: Overlay of various mean diameters over time

Converting the NanoSight number mean into an intensity mean utilises all the diameters seen from the NanoSight – this is not the case for the DLS, where the larger particles dominate. The 95th percentile of each time point from the NanoSight data is closer to the mean given by DLS, highlighting the inherent bias of DLS towards more strongly scattering particles. The NanoSight instrument therefore gives more information on the particle size distribution compared to that obtained from the DLS.

2.5 Conclusions

The NanoSight instrument and software were successfully used to analyse the particle growth of a poorly water soluble drug at a low (μM) concentration. The instrument offers an advantage over DLS for this particular sample in that the emergence of a polydisperse distribution over time can be seen. The results from DLS do not show this level of detail, with mean particle diameter only seen to increase over time. The NanoSight is however more difficult to use than DLS due to the numerous parameters for both video capture and processing which can be altered, and a certain amount of expertise is required. This is also acknowledged in the work of Filipe et al.[17]

The results from both light scattering techniques demonstrate that precipitation and particle growth of lipophillic molecules at low concentrations can be slow, occurring over several hours. This information is of practical relevance to drug screening.

Acknowledgements

With thanks to;

Alan Kennedy and Alastair Florence, (University of Strathclyde), for their comments on the paper

Ken Cameron (MSD Research, Newhouse, Lanarkshire) for performing the ^{1}H-NMR analysis

Jonathan Smith (NanoSight Technology Ltd) for technical advice on the instrument

MSD Ltd and the University of Strathclyde for funding contributions

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3. Detailed study of precipitation of a poorly water soluble test compound using methodologies as in activity and solubility screening - mixing and automation effects

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3.1 Introduction

Within the pharmaceutical industry, storage of discovery compounds dissolved in dimethylsulfoxide (DMSO) is widespread. The DMSO stock solution is then used as appropriate in several different analyses, some of which involve addition of a small aliquot of DMSO stock solution to an aqueous media. However, most drug molecules synthesised by medicinal chemists are largely water insoluble;[1] this trend and the reasons behind it have been discussed by Lipinski *et al*.[2] It has been acknowledged that precipitation of the compound could occur upon addition into the aqueous buffer.[3] This precipitation process and how it proceeds has implications for two important areas of analysis; activity assays in a high throughput screening (HTS) setting and kinetic solubility assays. Both of these analyses involve the addition of the test compound dissolved in DMSO to an aqueous buffer system. In an activity screening context, it is generally assumed that the compound remains in solution for the entirety of the analysis. However, given the low aqueous

solubility of many test compounds, precipitation is a strong possibility. This could potentially affect the in-vitro assay in two ways;

1. A false negative result could be obtained if a compound which has biological activity has not remained in solution and is therefore not available to interact with the enzyme/cell. This negative result obtained is caused by lack of solubility rather than a lack of biological activity.[4]
2. A false positive result could be obtained due to non-stoichiometric, aggregate based inhibition,[5] resulting from interaction of the precipitated particles with the enzyme. This means the compound has no specific molecular interaction with the enzyme in question, and the positive result is not due to stoichiometric biological activity. This phenomenon been discussed in depth by Seidler *et al*, McGovern *et al* and Feng *et al*.[5-9]

At the low concentrations and volumes used in HTS, it is difficult if not impossible to visually detect precipitation, particularly if small aggregates are formed. This means that potentially useful drugs could be discarded. These solubility issues in HTS have led to the introduction of what is known as 'kinetic' solubility assays. [3] As with activity screening, these tests also utilise the addition of DMSO stock solutions of the drug to an aqueous buffer, however here precipitation is an expected phenomenon. The assays are used to determine a guideline aqueous solubility value for the compounds under test. They give an indication of potential solubility issues early on and can be used to aid bioassay result interpretation.[3, 10] Investigation into the precipitation that occurs on mixing DMSO stock solutions and buffer, and the factors affecting the process, is therefore of interest to these two important areas of drug discovery analysis.

Several controllable factors could potentially affect the rate at which a compound precipitates from solution. The technique used for initial addition of the DMSO may be important, as well as the method for subsequent mixing. Within an activity assay setting, no particular importance is given to how the compound stock solutions in DMSO are added into the aqueous buffer, with many papers either stating that the

solutions were 'mixed' with no specific details, or not mentioning mixing at all. Within a kinetic solubility setting, some limited investigation into the effect of mixing has been carried out. Bard *et al* investigated the effect of shaking on precipitation, and found that more vigorous shaking resulted in lower solubility values (so long as the compound hadn't precipitated completely and some compound was still dissolved in solution at time 0).[11] Alelyunas *et al* state that 'stirring is one of the most important experimental variables in determining solubility' and noted stirring is likely to affect the supersaturation obtained during kinetic solubility assays.[12] Bevan *et al* however found that shaking is not required for reproducible results using their light scattering kinetic solubility determination method.[13]

The work reported here was conducted using a poorly soluble test compound (tolnaftate) to evaluate precipitation when DMSO stock solution and an aqueous buffer are mixed together at low concentrations (within the range used in HTS). Whilst several publications have looked at the kinetic solubility of many different compounds, the experiments here focus on exploring in detail the effect of different mixing variables on the precipitation and kinetic solubility of one model compound. The results here are intended as an initial indication of whether mixing and pipetting variables have an effect on the process at low concentrations and small scales. The use of robotics allows control over how the DMSO stock solutions and buffer are pipetted, with respect to the angle, speed and height used, and also mimics the procedure used in drug screening, where robotic liquid handling is common. Different mixing methods were investigated, and a comparison of the reproducibility of results obtained from robot and hand pipetting was performed.

3.2 Materials and Methods

The experiments were deliberately performed in 3 separate laboratories; Lab 1 (MSD Newhouse), Lab 2 (MSD Hoddesdon) and Lab 3 (University of Strathclyde).

Tolnaftate was purchased from Sigma-Aldrich, and its purity confirmed by NMR (>99%).

Dimethyl sulfoxide (DMSO) used in the experiments was HPLC grade from Sigma Aldrich (Lab 1), ACS reagent from Sigma Aldrich (Lab 2) or UV Vis grade from Fluka (Lab 3).

Buffer Reagents – KH_2PO_4 was purchased from AnalaR (Lab 1) or Fisher Scientific (Lab 2 and 3). Na_2HPO_4 (anhydrous) and $Na_2HPO_4.2H_2O$ were purchased from Fisher Scientific (all labs).

3.2.1 Stock and Buffer Preparation

Stock solutions of nominal concentrations 1 mM and 5 mM were prepared (actual stock solution concentrations ranged from 0.99 to 1.03 mM, and 4.97 to 5.08 mM). These solutions will be referred to by their nominal concentrations. An aliquot of all 1 mM stock solutions prepared was diluted with DMSO to give a 0.5 mM stock solution. Aliquots of the 5 mM stock were diluted to 2, 3, and 4 mM using DMSO. This gave a total of 6 stock solutions, originating from two weighed stocks. A pH 7.0 (±0.1) phosphate buffer was prepared, using 0.0025 moles of $KHPO_4$ and 0.0025 moles of Na_2HPO_4 per litre of deionised, Milli-Q water. The pH adjustment was conducted with 2 M NaOH if required.

3.2.2 Instrumentation

A summary of the instrumentation is given in Table I. Full details of individual HPLC methods and robot system settings are present in the appendix (chapter 3 additional data).

Table I: Instrumentation Summary

	Lab 1	*Lab 2*	*Lab 3*
Robot system	Tecan Genesis RSP 200	Beckmann Coutler Biomek 2000	N/A – samples hand pipetted
Robot system software	Genesis v. 4.217.304	Bioworks run version 3.5(2003)	N/A
HPLC system	Agilent 1100	Agilent 1100	Waters Alliance
HPLC column	Zorbax SB-C18 Rapid Resolution HT 2.1x50 mm 1.8 µm column	Phenomenex Luna C18(2) 50 x 4.6 mm 3 µm column	Thermo Scientific Hypersil Gold 50 x 4.6 mm, 3 µm column.
Drug retention time (mins)	2.44	2.18	1.99
Vortex used	MSI Minishaker IKA 2500 rpm	Eppendorf thermomixer comfort, 1.5 mL holder, 1400rpm	Vortex Genie 2, vortex setting 4 (around 1900 rpm)
Stirrer used	Heidolph MR3002	N/A	N/A
Centrifuge used	Eppendorf 5804, with T60-11 rotor	Eppendorf 5804 R, with F45-30-11 rotor	Eppendorf 5254 R with F45-24-11 rotor.
Average lab temperature (°C)	20	23	20.5

3.2.3 Sample Preparation using automated systems

Suspensions (test samples): an aliquot (10 µL) of 1-5 mM DMSO stock solution was pipetted by the robot into a vial. The pH 7 buffer was then added by the robot in two 495 µL aliquots (Tecan) or one 990 µL aliquot (Biomek). This gave 5 sample suspensions within a concentration range of 10-50 µmol/L (10-50 nanomoles/mL).

HPLC standards: an aliquot (10 µL) of 0.5-5 mM DMSO stock solutions was pipetted by robot into a vial. DMSO was then added in two 495 µL aliquots of DMSO (Tecan) or one (990 µL) aliquot (Biomek). This gave 6 calibration solutions in DMSO in the concentration range 5-50 µmol/L (5-50 nanomoles/mL). Fresh calibration solutions were prepared for every HPLC experiment.

The difference in pipetted volumes between the two instruments was due to the volume capabilities of the robot – the Tecan instrument had a maximum volume of 500 µL.

3.2.4 Sample preparation by manual pipetting

The same procedure as for robotic pipetting with the Biomek was followed, but with the samples being manually pipetted using Gilson pipetteman pipettes.

3.2.5 Mixing

After preparation, the samples were mixed as follows;

Vortex Mixing (VOR) – The samples were vortexed for 30 seconds.

Robot Aspiration Mixing (ASP-R) and Manual Aspiration Mixing (ASP-M) - After dispension of buffer, the robot aspirated and dispensed 500 µL of the suspension 5 times. The manual version of this mixing (ASP-M) was performed in a similar way i.e. around half the suspension was aspirated and dispensed 5 times after the addition of the initial buffer aliquot.

No Additional Mixing (NO) – After addition of the final aliquot of buffer, no further mixing was performed.

3.2.6 Centrifugation and Dilution

Immediately after the mixing stage (section 3.2.5), the samples were centrifuged for 5 minutes at 7200 x g rcf. Slightly different procedures were then used in each laboratory:

Lab 1 (initial samples prepared in a 1.5 mL, glass HPLC vial). 0.5 mL of the supernatant was removed and diluted to 1 mL with DMSO. The solution was vortexed for around 5 seconds at 2500 rpm to ensure thorough mixing. This is the supernatant sample. 1 mL of DMSO was added to the remaining 0.5 mL of solution plus any solid precipitate, giving a total volume of 1.5 mL. The mixture was vortexed for around 5 seconds at 2500 rpm, and placed on a rotator for 15 minutes at 20 rpm to allow full dissolution of any precipitated solid. This is the precipitate sample.

Lab 2 and 3 (initial samples prepared in a 1.5 mL, plastic microcentrifuge tube). The entire supernatant was removed from the tube, and 0.5 mL of supernatant was diluted to 1 mL with DMSO to give the supernatant sample. To the microcentrifuge tube containing the precipitate, 1 mL of DMSO was added, and the solution vortexed for around 5 seconds to ensure full dissolution of the precipitated material. This is the precipitate sample.

The samples were then analysed by HPLC. Lab 1 precipitate samples were adjusted for dilution factors, and by subtracting the amount of material contained in 0.5 mL of supernatant.

Recoveries were calculated by summation of the amount of drug present in supernatant and precipitate sample, and comparison with the amount of drug present in the initial sample. Experimental results with calculated recoveries lower than 85% or larger than 115% were rejected.

Mean results from experiments performed under various conditions in each lab were compared using t-tests in Microsoft Excel. This test returns a value of the probability that the two means come from two underlying populations with the same mean. 95% confidence level was used in all analysis. Equal/unequal variance t-test was chosen based on f-test result, which was also performed in Excel. Boxplots were created using MiniTab (v16).

3.2.7 NTA and NanoSight LM10 Analysis

For NTA (software version 2.1 build version 0316) analysis, the buffer was filtered (0.2 um filter), but otherwise the samples were prepared as described above. After mixing, samples were either incubated for 5 minutes or centrifuged for 5 minutes, and then introduced into the NanoSight LM 10 chamber. No dilution was performed. Video capture settings were shutter, gain 26, 0 and 153, 0 for incubated and centrifuged samples respectively. Processing parameters were kept as 'auto', with blur increased to 5 x 5. The instrument temperature was set to 25 °C. The resulting particle size distribution was that which had not been smoothed, obtained by pressing 'Ctrl + 0' during analysis. The distributions were then binned (20 nm).

3.2.8 Variation present in separation and dilution steps

A scaled up 50 µmoles of drug per litre of mixture suspension was prepared, by manually pipetting 200 µL of the 5 mM stock solution into a 30 mL glass vial. 19.8 mL of the pH7 buffer was added, and solution stirred using an 'x' shaped magnetic stirrer bar. One experiment was performed with a stirring speed of 375 rpm, and two with a stirring speed of 750 rpm. After stirring for 15 minutes, several 1 mL aliquots were removed (ranging in number from 10 to 18, depending on the experiment), and were placed into HPLC vials. The solution was stirred continuously during sampling. The samples were then centrifuged and diluted as those from the mixing experiment (section 3.2.5).

3.2.9 Kinetics of precipitation

Using the Tecan robot with the settings described previously, 20 test samples (at a concentration of 30 nanomoles of drug per mL of suspension) were prepared. The samples were all rotated for 5 minutes at 20 rpm. Five samples were centrifuged immediately; these are t=0. The remaining samples were incubated at 20 °C (± 2 °C), and at t=48 h, t=120 h and t=168 h, 5 samples were removed, centrifuged and diluted. Here, an increased centrifugation speed was used (10,000 x g rcf, 5 minutes).

3.2.10 Dissolution from solid

100 mL of a 1% DMSO/99% pH 7 buffer solution was added to approximately 1 mg of solid tolnaftate, resulting in a sample concentration of ca. 30 µM. The resulting suspension was stirred for 40 minutes at 500 rpm. 5 x 1 mL aliquots were then removed and centrifuged for 5 minutes at 10,000 x g rcf. 0.5 mL of the supernatant was removed and diluted twofold with DMSO. Only the supernatant samples were analysed in this experiment. The remaining mixture was kept stirring at a constant speed and the sampling process repeated. The sampling process was repeated after 24, 48 and 162.5 hours of stirring.

3.3 Results and Discussion

3.3.1 Dissolution from solid/kinetics of precipitation

Detailed results from the amount of drug in the supernatant after dissolution from solid and after prolonged incubation in the precipitation kinetics experiments are shown in the appendix. Whilst no specific value has been obtained from these particular experiments, both results sets agree that the true equilibrium solubility of tolnaftate is likely to be extremely low (around <2 µmoles of drug per L of 1% DMSO/99% buffer mixture). The initial time point for the precipitation experiment is higher than that starting from the solid. However, as the sampling time is increased, both solubility values tend to low values. Shorter timeframes are likely to be necessary to fully study the kinetics of precipitation, as it occurs rapidly in this compound. It is certain, however, that at the total drug amounts investigated here (30 and 50 µmoles of drug per L), the drug is way in excess of the solubility from the solid value, samples have high supersaturation, and precipitation is expected. It should however be noted that it is known that tolnaftate forms nanoparticles in the concentration range used here[14] and it is likely that centrifugation has not completely removed all of these nanoparticles from the supernatant. The amount of drug detected in the supernatant is therefore the sum of molecularly dissolved tolnaftate plus any nanoparticulate material which has not been centrifuged out.

3.3.2 Variation present in separation and dilution steps

To establish how much variability resulted from the variation inherent in a nucleation process, as well as variations in how the robot pipetted and mixed, it was necessary to first assess the reproducibility of the unavoidable manual steps involved in the experiments. This included both the removal of the supernatant after centrifugation and the dilution of samples for HPLC analysis. Solid tolnaftate could stick to the polypropylene pipette tip used whilst sampling due to both the compound and the tip being hydrophobic. This was in fact observed during

dissolution from solid experiments, and would affect the recoveries seen. For the reproducibility experiment, all replicate supernatant values from the same solution were used, even if total recoveries were low, as the adsorption of any solid drug to the pipette tip would not affect the amount seen in solution, only the total recovery. All samples came from the same homogeneous solution, and so the amount present in solution should therefore be the same for each sample. The results from these experiments show that, for a given sample where several aliquots are removed, the results are reproducible (Table II).

Table II: Reproducibility of manual steps

Treatment	Amount of tolnaftate in supernatant (µmoles per L)	
	Mean	***S.D.***
Stir 375 rpm	10.8	1.4
Stir 750 rpm R1	17.3	2.5
Stir 750 rpm R2	5.2	2.5

When different treatments were used, it can be seen that the standard deviation is of the order of a few nanomoles per mL.

3.3.3 Decrease in supernatant amount measured as more experiments performed

Table III shows the results of some experiments obtained from labs 1, 2 and 3. When performing the very first set of experiments, it was noticed that the standard deviation was higher than that obtained for the results in section 3.3.2. As several subsequent replicates were performed, the standard deviation further increased. Upon analysis of the full dataset, it was found that experiments performed early in the experimental time period had significantly higher amounts of tolnaftate present

in the supernatant with larger standard deviations than those performed later on (regardless of the mixing used).

In order to investigate this further, and to confirm this to be a real effect for this drug, the experiments were repeated in Lab 2 and Lab 3. Lab 2 had no recent history of usage of tolnaftate, and the stock solutions were prepared in Lab 3, so that no nuclei in the form of solid crystalline tolnaftate were introduced into the local environment by opening the bottle to weigh out the compound. Again, a similar trend was observed. Lab 3 also demonstrated this trend as for a group of mixing experiments performed over a non-continuous 5 month period, the amount of tolnaftate present in the supernatant in later experiments was lower. The division of results into 'initial' and 'further' experiments was arbitrarily performed by inspection of the data.

Table III: Comparison of Labs 1, 2 and 3, initial and further experiments amount of tolnaftate present in the supernatant

Treatment Details		Amount of tolnaftate in the supernantat ± S.D. (µmoles per L)		p value for t-test comparing early and later experiments
Lab and initial amount added	**Mixing**	***Initial experiments*** [a]	***Further experiments*** [b]	
Lab 1, 30 µmoles per L	NO	15.7 ± 5.6	6.1 ± 1.1	0.008
	ASP-R	12.7 ± 5.4	6.5 ± 1.7	0.02
Lab 1, 50 µmoles per L	NO	19.2 ± 5.6	9.6 ± 1.2	0.008
	ASP-R	14.6 ± 6.6	7.3 ± 1.2	0.07 (N.S)
Lab 2, 30 µmoles per L	NO	10.2 ± 1.1	9.2 ± 0.8	0.2 (N.S)
	ASP-R	5.7 (only 2 reps)	7.7 ± 1.9	0.2 (N.S)
Lab 2, 50 µmoles per L	NO	17.5 ± 1.2	7.7 ± 1.4	0.00002
	ASP-R	13.4 ± 5.1	5.7 ± 2.9	0.01
Lab 3, 50 µmoles per L*	NO	27.0 ± 1.1	10.7 ± 4.4	0.0005
	VOR	8.2 ± 1.5	4.5 ± 1.4	0.008
	ASP-M	15.0 ± 1.7	8.8 ± 2.9	0.01

[a] *– Lab 1 - Days 1-5*

Lab 2 - Day 1

Lab 3 – Day 1-2

[b] *–Lab 1 - Day 6 onwards*

Lab 2 - Day 2 onwards

Lab 3 – around 5 months later

**All experiments performed at 25 ± 1 °C, and centrifuged at 9,300 x g rcf. These are the only experiments to be performed under these conditions and were not used in comparisons to Labs 1 and 2.*

NS – not significant, p value >0.05

8 out of 11 t-tests show significant differences between mean supernatant values obtained at the start of the experimental time period and that obtained later in the

experimental time frame. The later values appeared to be fairly consistent, with less variation (judged by standard deviation values) than for earlier repetitions. The later values are also more in line with the standard deviation values seen in section 3.3.2.

Several aspects of the experimental procedure were examined for potential causes to this unusual result. Water uptake from DMSO stocks was investigated. All calibration solutions were prepared from the same stock solutions as the samples, thus any change in stock concentration due to water uptake would be noticeable in the peak areas of the calibration samples during HPLC analysis. This was not observed. The calibration lines themselves were also examined, and no changes in the slope and intercept were observed which would explain the effect. The trend could not be correlated to the age of the buffer used, or whether a freshly prepared stock solution was used – once the decrease had occurred, the supernatant amounts remained low despite preparation of fresh buffer/stock solutions. The experiments were performed at a constant temperature throughout. Potential explanations include the generation of homogeneous nuclei of the drug as more precipitation experiments were performed, resulting in increased levels of air borne nuclei of the drug. Something within the particular lab environment could also have changed, giving rise to heterogeneous nuclei (e.g. increased numbers of dust particles, which could act as nuclei, although this would have to have occurred at all three sites). Both types of nucleation would result in increased precipitation levels. These explanations are, of course, speculative.

For the 50 µmol/L samples with no mixing, a boxplot gives a pictorial representation of this trend over time for all three laboratories (Figure 1)

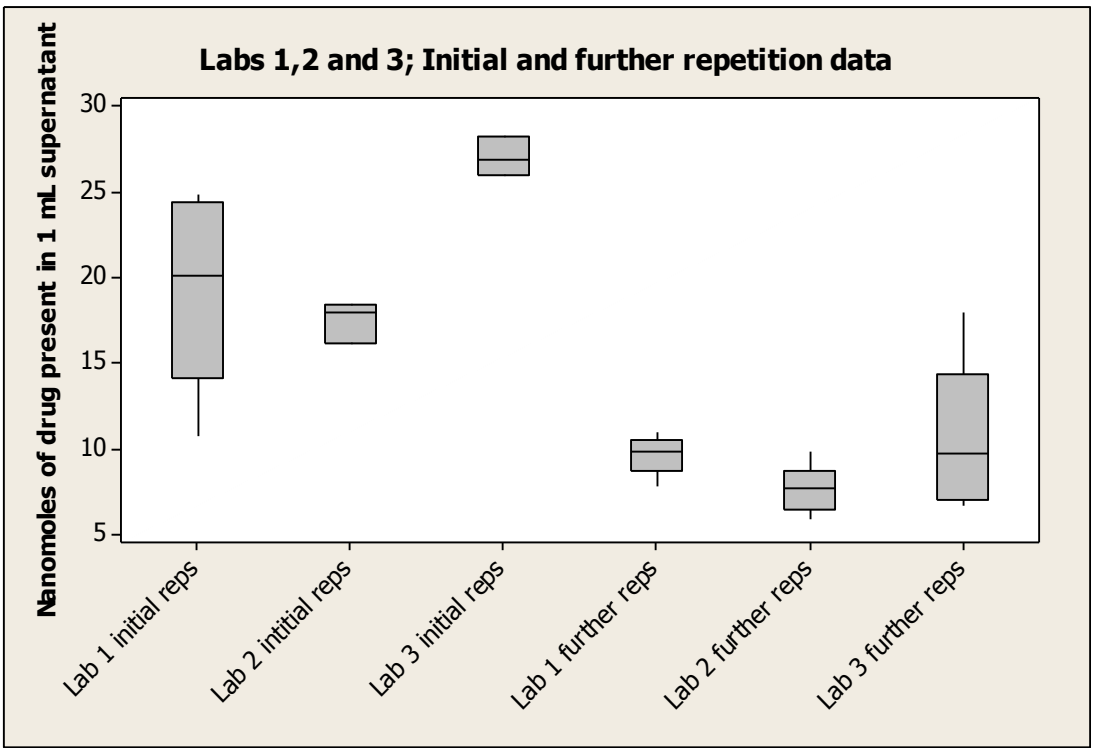

Figure 1: Tolnaftate 50 µmoles per L (50 nanomoles per mL) samples with NO mixing – comparison of initial and further reps for Labs 1, 2 and 3

In further comparison of the data only the results from the later experiments will be considered as they have been judged to have reached a steady, low value which remains unchanged as further experiments are performed. Although these later data sets tend to have less variability, they also have lower amounts of drug present in the supernatant. The difference between the earlier and later experiments can be up to 15 nanomoles in a 1 mL total volume – in the context of the low concentrations and volumes used in screening, this is a large portion of the total drug present. From the results seen here, further repetitions of a bioassay/kinetic solubility assay of this particular compound would give different results to those seen from an initial analysis.

3.3.4 Results and comparison of data between Labs

Presented in Table IV is the mean drug amount present in the supernatant (plus or minus the standard deviations) for all three labs (only further experiments shown). The measurement performed is essentially that of the kinetic solubility of tolnaftate under various conditions. This data will be used in comparisons between the labs, and also between different mixing procedures (section 3.3.6).

Table IV: Mean supernatant amounts (µmoles/L) with standard deviations

	Lab 1		Lab 2			Lab 3		
	NO	**ASP-R**	**NO**	**VOR**	**ASP-R**	**NO**	**VOR**	**ASP-M**
30 µmol/L	6.1 ± 1.1	6.5 ± 1.7	8.1 ± 1.3	5.9 ± 2.4	7.6 ± 0.9			
50 µmol/L	9.6 ± 1.2	7.3 ± 1.1	7.2 ± 1.5	4.9 ± 1.8	7.5 ± 3.0	9.3 ± 0.9	5.9 ± 1.0	7.9 ± 1.4

Lab 1- 30 nmol/mL, NO and ASP-R, n =14 and 10 respectively. 50 nmol/mL, NO and ASP-R, n=5 and 16 respectively. Lab 2 – n = 24 for all treatments. Lab 3 – n=9 for all treatments

Performing t-tests on the means obtained across different laboratories gives the p-values reported in table V.

Table V: Comparison of results between different labs; P values obtained after t-test

	NO mixing			VOR mixing	ASP mixing		
	Lab 1 vs. Lab 2	**Lab 1 vs. Lab 3**	**Lab 2 vs. Lab 3**	**Lab 2 vs. Lab 3**	**Lab 1 vs.Lab 2**	**Lab 1 vs. Lab 3***	**Lab 2 vs. Lab 3***
30 µmol/L	***0.00006***	**-**	-	-	0.08 (NS)	-	-
50 µmol/L	***0.002***	0.6 (NS)	***0.002***	0.1 (NS)	0.8 (NS)	0.3 (NS)	0.6 (NS)

**ASP-R vs. ASP-M*

NS – not significant at a 95% confidence interval

From table V, it can be seen that comparable supernatant amounts were observed for all three labs when VOR or ASP mixing were used. NO mixing, however, results in significant differences in 3 out of 4 comparisons (***bold and italicised***). This could be attributed to external laboratory factors (air borne nuclei) and/or the exact instrumentation used (e.g. initial dispension speed). It appears that the extent of precipitation depends upon the lab environment or the exact pipetting parameters used when no mixing is applied while any further mixing overrides this effect. This means that mixing of the samples should result in less lab to lab variation, provided they are mixed in a similar way – this result is somewhat intuitive.

Finally the results also show that manual aspiration mixing (ASP-M) is not significantly different from automated aspiration mixing (ASP-R). Control of the exact speed, height and angle that the buffer is dispensed/aspirated at does not significantly affect supernatant levels and these are similar to those for manual dispensing/aspiration.

3.3.5 Comparison of variability seen in robotically and manually pipetted samples

From the standard deviations present in the results (Table IV), there does not appear to be a significant advantage in using a robotic system to reduce variability. Obviously, this is not the reason why robotics are used for HTS, but it is an interesting point to note, and suggests that the variability present does in fact reflect the natural variation present in a precipitation process, rather than being caused by a controllable pipetting variable.

3.3.6 Comparison of mixing variables

Using the data detailed in Table IV, mixing variables were compared to one another and p values were obtained (Table VI).

Table VI: P values for mixing method comparison

	Lab 1	**Lab 2**			**Lab 3**		
	NO vs ASP-R	**NO vs VOR**	**NO vs ASP-R**	**VOR vs ASP-R**	**NO vs VOR**	**NO vs ASP-M**	**VOR vs ASP-M**
30 µmol/L	0.5 (NS)	***0.001***	0.2 (NS)	***0.007***	-	-	-
50 µmol/L	0.0008	***0.00004***	0.7 (NS)	***0.002***	***0.000007***	0.02	***0.002***

NS – not significant at a 95% confidence interval

There is no particular correlation between the amount of drug present in the supernatant and whether automated pipetting or manual pipetting is used (Table IV and

VI): results for Lab 1 NO mix (robotic pipetting) and Lab 3 NO mix (manual pipetting) are similar. Control of the exact pipetting parameters used therefore does not significantly affect the results seen, and provided further mixing is performed, lab to lab variation is minimal.

From the data, VOR mixing consistently leads to amounts of tolnaftate present in the supernatant (***bold and italicised***) which are significantly lower from that for other mixing types, for all three labs. Data at 25 °C from Lab 3 (shown in appendix) also demonstrates this trend. Overall it can be concluded that NO and ASP-R/M mixings tend to be fairly similar with significant differences not seen consistently (i.e. 3/5 comparisons not significant, 2/5 comparisons significant). However VOR mixing always results in a lower amount of tolnaftate present in the supernatant than for other mixing types investigated. As mentioned previously, it is known that these mixtures form nanoparticles of the drug.[14] The nanoparticles are present almost immediately upon addition of the buffer to the DMSO stock i.e. the induction time for these particles to form is extremely short. After addition of the buffer aliquot (be it by robotic means or manually), it is expected that these nanoparticles are already present in fairly high abundance. Subsequent mixing would therefore affect any further growth and ripening of these particles, rather than initial nucleation. Vortex mixing is the most vigorous mixing method used, and appears to be aiding in particle growth and/or ripening, facilitating the formation of larger particles compared to those formed with other mixing types. A more vigorous mixing method would also result in increased likelihood of collisions between particles, and aggregation of the particles could potentially occur. All of these scenarios would result in increased numbers of particles being removed during centrifugation, and so leaving less in the supernatant.

3.3.7 NTA Particle size analyses

In order to test the hypothesis offered in section 3.3.6, 50 µmoles per L VOR and NO mix samples were analysed using NTA. Samples were analysed both after 5 minute

centrifugation (analysis of the supernatant) and with a 5 minute incubation period (analysis of the suspension without prior separation of solid and liquid phases).

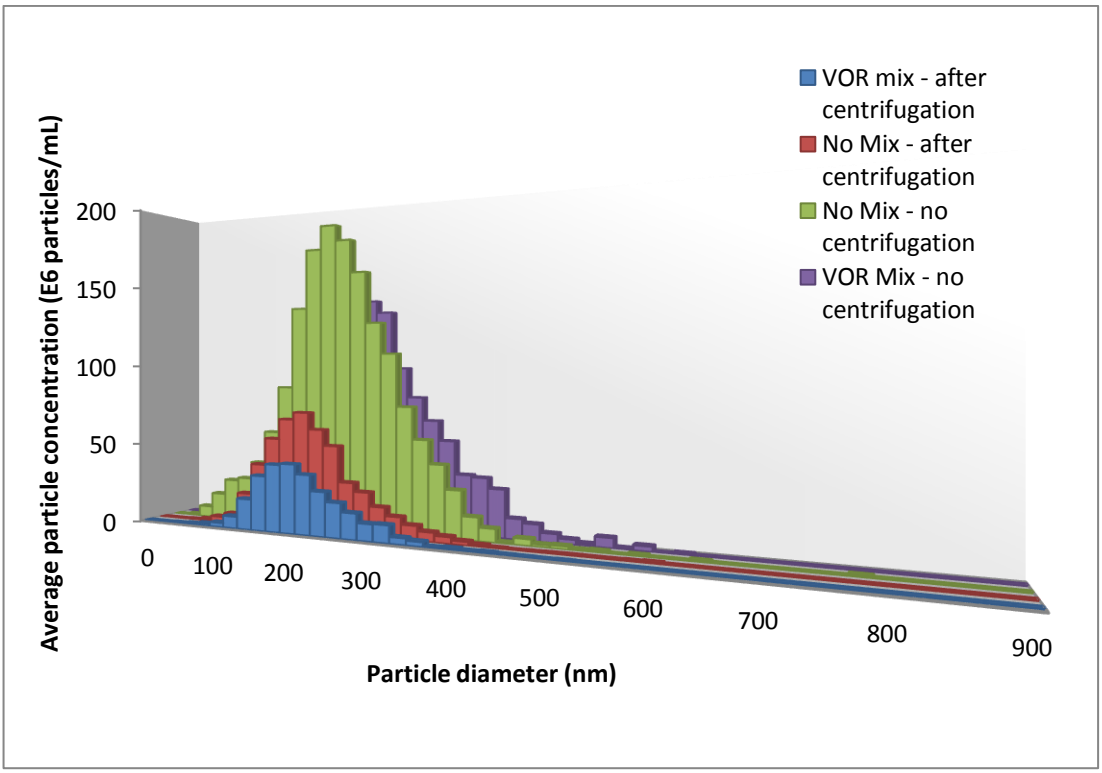

Figure 2: NTA Data; samples incubated for 5 minutes or centrifuged for 5 minutes at 9,300 x g rcf

Prior to centrifugation, both NO and VOR mixed samples give very similar mean particle diameters and particle concentrations. The particle size distributions are also fairly similar, although it can be seen that the VOR mixed samples have slightly higher concentrations of particles >300 nm. This is consistent with the view that more vigorous mixing affects the particle size distribution, although the effect is minimal. After centrifugation, however, a large difference is seen, suggesting centrifugation itself consolidates the effect seen from mixing. NO mix samples have a higher particle concentration remaining in the supernatant compared to VOR mixed samples, and also have a greater concentration of larger particles (>200 nm). Whilst the NTA concentration output is an estimate, based on 5 replicates, the nanoparticle concentrations for NO mixed and VOR mixed after centrifugation are significantly different ($p = 0.02$). This is consistent with the HPLC data, where NO

mixing results in a higher supernatant content – the NTA data suggests that this is not due to an increase in molecularly dissolved content, but an increase in nanoparticulate content which is not removed by centrifugation compared to those mixed by vortex.

3.4 Conclusions

Several conclusions can be drawn from the work presented. Experiments performed early in the experimental time period have a significantly different (higher) mean supernatant amount (with increased standard deviations) compared to those performed after a given time. This decrease has been observed at three different laboratories, confirming it as a real effect for this particular compound. Whilst it cannot be completely rationalised, it is of practical relevance to screening – further repetitions of this compound's kinetic solubility/bioassay activity would result in significantly different results to those seen from an initial screen.

No significant differences are seen between manual and robotic pipetting, in terms of either the amount of drug present in the supernatant or the variability seen from the samples. These data are useful in trying to understand the factors which affect the precipitation of poorly soluble drugs from these types of samples, and gives more insight into the process – the use of robotics does not decrease the standard deviations seen.

It can be concluded that whilst the way in which the initial addition of the organic and aqueous components occurs is not particularly important, subsequent mixing is. More vigorous mixing consistently gives lower supernatant concentrations than the other two mixing types investigated here, and affects the concentration of precipitate nanoparticles remaining after centrifugation. Again, this has implications for screening and kinetic solubility assays, where sometimes no particular importance is given to how the samples/plates are mixed.

Performing similar experiments with other compounds would aid in further understanding of the effect of mixing and pipetting variables on the precipitation process as a whole, but initial results here suggest mixing is a potential source of variation in the bioassay/solubility screening procedure which is not currently controlled.

Acknowledgements:

Alastair Florence (Univ. Strathclyde) and Timothy Rhodes (Merck) for their useful discussions on the experiments.

Ken Cameron (MSD Research, Newhouse, Lanarkshire) for performing the ^{1}H-NMR analysis

University of Strathclyde and MSD for funding contributions

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4. Investigating the effects of nanoparticle formation on the kinetic solubility determination of 3 hydrophobic test compounds

4.1 Introduction

One of the most important physical properties of a candidate drug within the pharmaceutical industry is aqueous solubility. For a given compound, it is investigated (to varying degrees) at several stages in the drug discovery and development process.[1] Whilst good aqueous solubility is a very desirable property, generally compounds synthesised by the industry at the discovery stage are poorly water soluble and hydrophobic. They are optimised structurally with a view to maximising binding to the target in question, and this is usually at the expense of aqueous solubility. Compounds tend to be selected for further investigation based on their affinity for their particular target - physical property investigation at this stage is minimal.[2] Moving on to a development setting, one of the big challenges facing scientists is to come up with the optimum salt/crystalline form/formulation which will give the final marketed product the desired physical properties. Detailed investigation of the physical properties of the compound and its formulations are therefore required.[3]

In a discovery setting, higher throughput solubility methods are used to give an initial indication of the solubility of the compounds under test. These analyses typically utilise DMSO stock solutions of the drug, short incubation times, and are said to be representative of the solubility of the fastest precipitating form.[4] It is not expected that types of samples reach an equilibrium solubility value. Two main methods of analyses are employed;

1. Using nephelometric[3,5] or turbidity based[4] methods to determine the concentration at which precipitation occurs. Here, DMSO stock is added incrementally to an aqueous phase until the solubility limit is reached.

2. Addition of the DMSO stock and aqueous phase, followed by separation of any solid formed (via centrifugation or filtration) and analysis of the filtrate by UV or HPLC.[3, 6, 7]

Analyses which utilise DMSO stock solutions of the compound are known generally as 'kinetic solubility' methods. These assays have several advantages; they utilise the drug stock dissolved in DMSO, making automation possible and so can be performed in 96, 384 etc well plates. Minimal compound is also used, which is essential at the discovery stage.[8] The samples do, however contain DMSO (sometimes varying amounts) in the final solvent composition, and it cannot be said for certain if the system has reached equilibrium. These analyses are, however, successful in giving an estimate of aqueous solubility of the compound, which is deemed sufficiently informative for the discovery stage.

In a development setting, solubility is determined thermodynamically, using a classical 'shake flask' approach. The compound is introduced in a solid form (in excess) into the solvent, and agitated for extended time periods, to ensure full dissolution to equilibrium.[1] Analysis is then performed after separation of the excess solid, typically by HPLC. This approach, whilst considered to give the 'true' solubility of a compound under a given set of conditions, is time consuming and requires larger amounts of compound compared to its kinetic counterpart.[8]

Typically the results from 'kinetic' solubility assays tend to be higher than those obtained from their thermodynamic counterparts. This is attributed to several different factors, such as the presence of small quantities of DMSO (which could increase solubility compared to aqueous systems alone – a so-called 'co-solvent' effect), the shorter incubation times employed (the system may remain supersaturated in the timescale employed), and the fact that the precipitating form may not be the thermodynamic crystalline form (amorphous/higher energy polymorphs can have increased solubility compared to the lowest energy polymorph).[1] All of these scenarios would lead to a higher solubility value than that obtained from a solid starting material in a 'shake-flask 'experiment. Approaches

have been made to try and minimise discrepancies between kinetic and thermodynamic solubility values, such as the use of longer incubation times[5] and the removal of DMSO to give a solid prior to solubility measurements, [9] whilst still retaining some of the advantages of 'kinetic' based analysis.

In the work described here, a detailed investigation into the thermodynamic and 'kinetic' solubility of 3 hydrophobic test compounds (amiodarone HCl 'ami', clotrimazole 'clot' and tolnaftate 'tol') was carried out. The behaviour was determined from the solid form using a 'shake-flask' type method and from DMSO stock solutions after precipitation using 1) light scattering (NTA and NanoSight) detection of particles and 2) HPLC analysis (after either centrifugation or filtration) with various incubation periods. As stated, typically analyses which utilise the drug dissolved in DMSO stock solution are referred to as 'kinetic methods'. Here, we will use the terminology 'precipitation' methods, to emphasise the physical process occurring. Since the solubility from 'shake-flask' (starting from solid) experiments used in comparison were obtained in solvent systems containing 1% DMSO, co-solvent effects were already accounted for when comparing to precipitation methods. The aqueous solubility was also predicted using the general solubility equation (GSE).[10]

4.2 Materials and methods

Amiodarone HCl (A-8423), clotrimazole (C-6019) and tolnaftate (T-6638) were purchased from Sigma-Aldrich. Compound purity confirmed by NMR, and either matched or exceeded the manufacturer's specifications. ($\geq$ 98%).

The DMSO used was UV-Vis grade, purchased from Fluka.

KH_2PO_4 and $Na_2HPO_4.2H_2O$ were purchased from Fisher Scientific.

Citric acid was purchased from Fisher Scientific.

4.2.1 DMSO stock and buffer preparations

Weighed stock solutions of nominal concentrations 1 mM and 5 mM were prepared in DMSO for each individual compound (actual stock solution concentrations ranged from 0.99 to 1.03 mM, and 4.97 to 5.08 mM). These solutions will be referred to by their nominal concentrations. Note that amiodarone HCl DMSO solutions visually began to degrade after ~10 days of storage at room temperature. Fresh stocks were therefore prepared weekly for this compound. Clotrimazole and tolnaftate stocks were monitored by HPLC and found to be stable for several weeks.

A pH 7.0 ($\pm$ 0.05) phosphate buffer was prepared, using 0.0025 moles of $KHPO_4$ and 0.0025 moles of Na_2HPO_4 per litre of deionised, Milli-Q water. The pH adjustment was conducted with 2 M NaOH if required.

A pH 4 citric acid/phosphate buffer was prepared by mixing 147 mL of a 0.1 M citric acid solution and 103 mL of 0.2 M sodium diphosphate solution and diluting to 500 mL with Milli-Q water. The buffer was adjusted using citric acid to pH 4.0 ($\pm$0.05).

4.2.2 'Shake-flask' based method of solubility determination

An excess of solid (~ 10 mg) was added to 100 mL of a 1% DMSO/99% buffer mixture (pH 4 for amiodarone HCl, pH 7 for clotrimazole and tolnaftate) and stirred at 25 °C using a Fisher scientific stirrer FB15001, setting 4.5, in a 250 mL vessel using a 4 cm length stir bar. At various time points (1 hour up to one week), 3 x 1 mL aliquots were removed and centrifuged at 9,300 x g rcf for 5 minutes using an Eppendorf 5254 R with a F45-24-11 rotor, set at 25 ± 3 °C. 0.5 mL of supernatant was removed and diluted to 1 mL with DMSO. The samples were then offered to HPLC for analysis. For amiodarone HCl samples, the solid suspension appeared very turbid, as if very small particles were present. Centrifugation gave variable results – thus, for this compound, 0.22 µm Whatman 4 mm syringe tip filters were used during 'shake-flask' experiments rather than centrifugation. Due to the low concentration of drug in present in aqueous solution, HPLC injection volumes for 'shake- flask' method samples were typically increased from those documented in table I. No trend was seen over time for any compound investigated – thus, an average value of all timepoints was taken.

4.2.3 GSE

The general solubility equation as defined by Sanghvi et al;[10]

$\text{Log } S_w = -0.01 (MP-25) - \log K_{o/w} + 0.5$

Equation 1

Where;

Sw = aqueous solubility, moles per litre.

MP – Melting point in °C

Log $K_{o/w}$ = log octanol/water partition co-efficient.

The values used for each compound found from literature shown in table I. All octanol/water co-efficient values are experimental LogD values at pH 7.4

Table I: Physical properties used in GSE calculation

Compound	*Melting point (° C)*	*Log Ko/w*
Ami	161[18]	4.92[19]
Clot	143.5[20]	4.9[21]
Tol	112[22]	5.4[9]

4.2.4 Precipitation method 1 - NTA determination of drug concentration at which nanoparticles are first detected

Various diluted drug stock solutions in DMSO (0 to around 2 mM, depending on the compound) were prepared from the 1 and 5 mM stock solutions detailed above. At all drug stock concentrations, 10 µL of stock was added to an HPLC vial. 990 µL of buffer of the appropriate pH (4 for amiodarone HCl, 7 for clotrimazole and tolnaftate) was filtered and added. The % DMSO by volume was kept constant at 1%. The samples were removed from the vial and introduced into the NanoSight chamber. The point at which the measured nanoparticle concentration became significantly different from that of the blank was determined in Excel using a two tailed t-test (95% confidence interval).

4.2.5 Precipitation Method 2 - Analysis by HPLC and subsequent investigation of separation and kinetics by NTA

(i) Suspension Preparation

An aliquot (10 µL) of 5 mM DMSO stock solution was pipetted into a microcentrifuge tube. A 990 µL aliquot of the appropriate buffer was then added. This gave a total concentration of 50 µM with a 1% DMSO content by volume.

For NTA analysis, the pH 4 or pH 7 buffer was filtered using a 0.2 µm hydrophilic PTFE filter (Millipore) before addition to the DMSO aliquot. Precipitations were also performed in glass HPLC vials rather than plastic microcentrifuge tubes for NTA. If centrifugation was required, the suspension was transferred using a glass pipette to a microcentifuge tube after initial precipitation had been initiated. All samples were prepared at 25 °C. A minimum of three replicates were performed for each variable.

(ii) Separation and Analyses

After incubation (if required), the samples were then either centrifuged (9,300 x g rcf, 5 minutes) or filtered (0.45 µm PVDF Millex-HV 4 mm syringe filter, 0.22 µm Whatman 4 mm syringe tip filter).

NTA – supernatant/filtrate samples were measured on the NanoSight as is. Note samples were also analysed by NTA with no separation performed i.e. immediately after addition of the buffer aliquot.

HPLC Analysis – Dilution and further preparation required.

For centrifuged samples - The entire supernatant was removed from the microcentrifuge tube, and 0.5 mL of supernatant was diluted to 1 mL with DMSO to give the supernatant sample. To the microcentrifuge tube containing the precipitate, 1 mL of DMSO was added, and the solution vortexed for around 5 seconds to ensure full dissolution of the precipitated material. This is the 'precipitate' for centrifuged samples.

For filtered samples – The filtrate was diluted with DMSO by a factor of 2. The used filter tip was retained, placed back into the tube where the initial precipitation had taken place, and 1 mL of DMSO added. The tube was rotated for 15 mins to ensure dissolution of the solid within the filter membrane. This is the 'precipitate' for filtered samples.

The samples were then offered to HPLC for analysis.

Addition of the quantified amounts from both the supernatant and precipitate gave the total amount recovered for each sample. Recoveries in the range 85-115 % were deemed acceptable and included in the calculation of the average. Filtration consistently gave total recoveries < 85% for all compounds – results are still displayed with this noted.

4.2.5 Instrument Details

(i) HPLC Settings

Analysis was carried out using a Waters Alliance HPLC system, separation module 2695, dual wavelength detector 2487 and PDA detector 2996, used in conjunction with Empower Pro, 2002 (build 1154) software. The column used was Thermo Scientific Hypersil Gold 50 x 4.6 mm (or 50 x 4 mm) with a 3 µm particle size. Mobile phase A comprised of Milli-Q ultra filtered water + 0.01 % TFA and Mobile phase B comprised of HPLC Grade MeCN + 0.01% TFA. For all compounds, the column temperature was set to 40 °C and the mobile phase flow rate to 1 mL/min. The total data collection time was 3 minutes for all 3 compounds and an isocratic method was used. Individual HPLC settings were as follows;

Table II: HPLC conditions

Compound	*Mobile phase ratio A:B (%)*	*Detection wavelength (nm)*	*Injection volume (µL)*	*Retention time (mins)*
Ami	60:40	270	10	1.82
Clot	60:40	265	10	1.77
Tol	40:60	257	3	1.99 (2.7 on 50 x 4 mm column)

Calibration procedure – 6 calibration solutions (100% DMSO) were prepared in the concentration range 5 to 50 µM for each compound, originating from 1 and 5 mM weighed stock solutions in DMSO as detailed previously. Fresh calibration solutions

were prepared and analysed for each experiment along with the samples under test.

(ii) NTA Settings

Instrument - NanoSight LM10 with NTA software version 2.1 release build 0316 (2010).

Videos lengths were 90 seconds, unless otherwise stated, and all processing parameters set to automatic. The blur was increased in all cases to 5 x 5. All analyses were performed at 25 °C. A distribution which had been adjusted as normal by the software, but had not been smoothed, was obtained. This non-smoothed data is that shown or used further in calculations etc. Camera shutter and gain settings for each compound and experiment are detailed in table III.

Table III: NTA Video capture settings

	Kinetic method 1 - NTA		*Kinetic Method 2 - Various separation techniques*					
			No separation		Centrifugation		0.45 Filtration	
Compound	***Shutter***	***Gain***	***Shutter***	***Gain***	***Shutter***	***Gain***	***Shutter***	***Gain***
Ami	1500	680	1500	500	1500	680	1500	680
Clot	1500	680	100	0	605	260	1500	680
Tol	700	690	66	0	153	0	300	459

The amount of drug present as particles can be estimated from the NanoSight output, by calculation of the volume mean and utilisation of the NTA concentration estimate. [23] Knowledge of the particle density is also required – here we used the density of the crystalline solid in the calculations, although accept it is not known whether the particles are in fact crystalline or not. (Density values used in the calculation are in g/cm^3 – Amiodarone HCl, 1.71,[24] Clotrimazole, 1.316[25] and Tolnaftate, 1.223[23]).

4.3 Results and discussion

4.3.1 'Shake-flask' solubility and GSE results

The results from the 'shake-flask' solubility experiment and GSE calculations were as follows (table IV);

Table IV: Solubility from solid and GSE values

Compound	*'Shake-flask' solubility ± S.D (µM)*	*GSE predicted solubility (µM)*
Ami	1.9 ± 0.2	1.7
Clot	4.3 ± 1.3	2.6
Tol	1.5 ± 0.8	1.7

Ami – n =15 (average of 5 timepoints, 3 samples per timepoint), Clot – n=9 (average of 3 timepoints, 3 samples per timepoint), Tol – n=15 (average of 5 timepoints, 3 samples per timepoint).

No corrections were applied for % ionisation (relevant for amiodarone HCl and clotrimazole) at the pH of solution used in the measurement. Amiodarone HCl and clotrimazole would be expected to be positively charged at the pH values used here (7 for clotrimazole and 4 for amiodarone HCl). All partition values used are LogD at pH 7.4. The GSE gives the solubility of the compound in pure water, whereas the 'shake-flask' experiments contain 1% DMSO. Despite all of these factors, the GSE gives solubility values which are in good agreement with those obtained experimentally from the solid using a 'shake-flask' method.

4.3.2 Methods originating from DMSO stock solutions – precipitation methods

(i) Precipitation Method 1 - NTA based

The first method used to determine solubility utilising DMSO stocks employed the NTA and the NanoSight to measure the increase in particle concentration as drug

concentration is increased. Test compound concentrations above and below the 'shake-flask' solubility were tested until concentration at which nanoparticle formation began could be determined (table V). This was performed by obtaining the x- intercept of the slope of increasing particle concentrations, in a similar way to that described by Hoelke *et al*.[3]

Table V: Kinetic solubility from NTA results

Compound	***Drug concentration at which particle formation occurs***
Ami	5 μM
Clot	16 μM
Tol	2 μM

In order for this analysis to give a similar result to that obtained from section 4.3.1, one main criterion would have to be met. The drug would have to precipitate as soon as the equilibrium solubility value was exceeded i.e. not have the ability to remain supersaturated for any length of time. The fulfilment of this criterion will of course be compound specific. For tolnaftate, the result in table V is in reasonable agreement with the results obtained from section 4.3.1, suggesting that despite using a precipitation based method, a solubility value very close to the equilibrium solubility has been obtained. Nucleation is thus initiated at total drug concentrations very close to the equilibrium solubility for this compound. For amiodarone HCl and, in particular, clotrimazole, the results are slightly higher than those from the 'shake-flask' emthod and GSE, suggesting these compounds have the ability to remain supersaturated for the short time period of the experiment. For all three compounds, nanoparticle formation occurs rapidly at concentrations fairly close to the equilibrium solubility value.

This approach to determining kinetic solubility has a similar underlying principle to the use of nephelometric methods of kinetic solubility determination i.e. light

scattering is used to detect the total drug amount at which scattering from precipitate becomes significant compared to background scattering. It has been stated however that nephelometric methods have a lower limit of around 20 µM.[7] Whilst the NTA method used here appears more sensitive, it does not have the capability to be automated or performed in plates. The results presented here have constant % DMSO, whereas in some other analyses, the % DMSO can vary as stock solution is added incrementally to a buffer until scattering is detected.[2]

(ii) Precipitation Method 2 - HPLC based

The second precipitation method involved adding a volume of drug dissolved in DMSO to the buffer such that the total drug amount was in excess of that expected to remain dissolved in solution. The same concentration (50 µM) was chosen for all three compounds. Since the system has a total drug content above the equilibrium solubility value, precipitation is expected to occur. After precipitation, the solid and liquid phases are separated and quantification of the amount of drug in each phase performed by HPLC. The results from these experiments are shown table VI;

Table VI: Kinetic solubility results for all three test compounds, with sample analysis performed by HPLC

Compound	Amount present in supernatant ± S.D. (µM), after centrifugation (5 mins, 9,300 x g rcf)	Amount present in supernatant ± S.D. (µM), after 0.45 µm filtration	Amount present in supernatant ± S.D. (µM), after 0.22 µm filtration
Ami	27.5 ± 1.53 (n=15)	27.3 ± 2.7 (n=6)	*11.9 ± 0.8 (n=3)
Clot	20.2 ± 3.2 (n=21)	*8.4 ± 0.6 (n=6)	N/A
Tol	13.3 ± 5.6 (n=30)	*No Peak Detected	N/A

**poor total recovery (<85%)* *N/A – analysis not performed*

Again, in order for the results obtained to be similar to those from section 4.3.1, certain conditions must be met. The drug must precipitate (within the timescale of the experiment) to its equilibrium solubility, and not remain supersaturated for any

extended time period. The separation process of the solid and the liquid phases must be efficient.

From table VI, various different results were obtained depending on the exact conditions used to separate the solid and liquid phases;

(i) Centrifugation

All three compounds show significantly more compound present in the supernatant after centrifugation than would be expected from the results in section 4.3.1. Tolnaftate shows the least amount of drug in the supernatant, and amiodarone HCl the most. The total amount of drug present per mL (50 nanomoles) is constant between compounds, however in order to fairly compare the relative drug amounts in the supernatant after centrifugation treatment, the 'apparent' supersaturation (total drug amount present/'shake-flask' solubility) should also be accounted for. This calculates as 33 for tolnaftate, 26 for amiodarone HCl and 12 for clotrimazole. Fast precipitation would be expected from all three compounds from these calculated values. Tolnaftate has by far the highest 'apparent' supersaturation, and as expected has precipitated the most within the experimental timescale (lowest supernatant amount). Clotrimazole has the lowest 'apparent' supersaturation, and could therefore be expected to have the slowest kinetics, with the highest supernatant amount. This is not the case, with amiodarone HCl having the highest supernatant content.

(ii) Filtration

For 2 compounds (clot and tol), 0.45 µm PVDF filtration lowers both the drug amount detected after liquid/solid separation and the % recovered compared to centrifugation. For amiodarone HCl, no difference is seen in either drug amount detected in the filtrate compared to the supernatant or in % recovery. Moving down to a smaller pore size filter (but maintaining the same membrane type) results in a lower amount of drug detected and lowered recoveries.

Overall, the results detailed here are significantly higher than those obtained from the solubility determination methods discussed earlier. As mentioned, 'kinetic solubility' assays can result in the formation of supersaturated solutions – this would explain the increase in solubility value seen if it were assumed that the compound had not reached equilibrium within the timescale of the experiment. There also had to be sufficient separation of the solid and liquid phases – this was investigated further (table VII). Note we can rule out immediately any co-solvent (thermodynamic) increase in solubility from the presence of DMSO itself as the 'shake-flask' experiments used in comparison were also performed in a 1% DMSO/buffer solvent system. Another complicating factor is that hydrophobic compounds have a tendency to adsorb non-specifically to filter membranes – this would account for the reduction in both detected drug amount and % recoveries seen for clotrimazole and tolnaftate after use of 0.45 µm PDVF filters. Amiodarone HCl however only showed a decrease in recovery and drug filtrate amount when 0.22 µm filters were used.

In order to further investigate these results, similar experiments were performed but this time followed by NTA rather than HPLC analysis, with results as detailed in table VII.

Table VII: NTA analysis of the supernatant/filtrate after sample suspension centrifugation/ filtration

	5 mins 9,300 x g cent			***0.45 um PVDF filter***		
Compound	**Diameter (nm)**	**Particle concentration (10^6 particles/mL)**	**Calculated nanomoles of drug per mL**	**Diamter (nm)**	**Particle concentration (10^6 particles/mL)**	**Calculated nanomole of drug per mL**
Ami	111 ± 2	12 ± 1	3 ± 0.3	110 ± 14	10 ± 7	4 ± 3
Clot	<100 tracks*	0.4 ± 0.2*	N/A	< 100 tracks*	0.2 ±0.2*	N/A
Tol	221 ± 9	5 ± 2	14 ± 6	< 100 tracks*	0.03± 0.06*	N/A

*<100 tracks – calculated diameter from NTA analysis inaccurate due to low number of particles included in distribution. This is also reflected in the particle concentrations obtained, which are very low.

It can now be seen from table VII that the centrifugation parameters used here do not completely remove the nanoparticles for all compounds under test, and that filtration has varied results. This helps explain the high results seen. Despite the presence of nanoparticles and the kinetic nature of the measurement, the results produced by HPLC were very reproducible as can be seen from the small standard deviation values in table VI. It is worth mentioning that several replicates of the centrifuged HPLC samples (table VI) were performed (15 for amiodarone HCl, 21 for clotrimazole and 30 for tolnaftate), using various different stock solutions, buffer solutions and on different days – the HPLC supernatant amounts are very consistent despite this. The results can now be rationalised on a compound by compound basis.

Table VIII summarizes the results from the various 'solubility' assessments detailed so far for tolnaftate;

Table VIII: Summary of results from Tolnaftate solubility experiments – 'shake-flask', GSE and precipitation based methods

	'Solubility' value obtained (µM - mean plus/minus standard deviation)				
	'Shake-Flask'	***GSE***	***Precipitation method 1 (NTA based)***	***Precipitation method 2 (centrifugation then HPLC)***	***Precipitation method 2, (0.45 µm filtration, then HPLC)***
Tol	1.5 ± 0.8	1.7	2	13.3 ± 5.6	no peak detected

**poor recovery*

Table VIII shows that tolnaftate's equilibrium solubility is low; however the 'solubility' value obtained from precipitation method 2 (centrifugation then HPLC analysis) however gives a much higher supernatant value. We can now attribute this to nanoparticles (table VII) which have not been removed by centrifugation. Thus, we are not seeing supersaturation in the usual sense, but a sample which contains nanoparticles contributing to the HPLC supernatant amount. From NTA calculations, the amount of drug present as nanoparticles more than accounts for the HPLC supernatant amount. It should however be borne in mind that this calculated amount is an estimate due to the nature of the NTA concentration measurement.

This also agrees with the precipitation and NTA analysis results, where tolnaftate shows little tendency to remain supersaturated. Upon filtration, the particles are completely removed and the HPLC response obtained comes from molecularly dissolved solution only. The measured response after filtration is below that of the solubility from solid value, likely due to non-specific adsorption of the drug to the filter membrane, which also explains the lower recovery values.

A summary of clotrimazole's behaviour is detailed in table IX;

Table IX: Summary of results from Clotrimazole solubility experiments – 'shake-flask', GSE and precipitation based methods

	Solubility value obtained, (µM - mean plus/minus standard deviation)				
	From Solid	***GSE***	***Precipitation, by NTA***	***Precipitation, centrifugation then HPLC***	***Precipitation, 0.45 µm filtration, then HPLC***
Clot	4.3 ± 1.3	2.6	16	20.2 ± 3.2	8.4 ± 0.6

Again, the precipitation method with HPLC analysis shows a much higher value compared to that obtained from solid. From table VII, clotrimazole shows very few particles present, either after centrifugation or filtration. A reduction in the detected drug and in the total recovery by HPLC is, however, seen after filtration, again suggesting some adsorption. Both filtered and centrifuged samples have a drug response higher than the solubility from solid value. From the filtered results, the compound shows the capability to remain supersaturated in the conventional sense at this concentration. This also agrees with the results in table V, where clotrimazole demonstrated a capability to remain supersaturated.

Amiodarone HCl's behavior can be summarized as follows;

Table X: Summary of results from Amiodarone HCl solubility experiments – 'shake-flask', GSE and precipitation based methods

	Solubility value obtained, (µM - mean plus/minus standard deviation)					
	From Solid	***GSE***	***Precipitation, by NTA***	***Precipitation, centrifugation then HPLC***	***Precipitation, 0.45 µm filtration, then HPLC***	***Precipitation, 0.22 µm filtration, then HPLC***
Ami	1.9 ± 0.2	1.7	5	27.5 ± 1.5	27.3 ± 2.7	*11.9 ± 0.8

*poor recovery

Again, the compound has low solubility, but this is not apparent from the precipitation-HPLC based experiments. However, whilst high concentrations of nanoparticles are present for amiodarone HCl, their small size means they are estimated to contribute to only a few µM of the measured HPLC response, (Table VII, NTA data). This is in contrast to tolnaftate, where the particles accounted for a substantial portion of the HPLC supernatant value. Filtration through 0.45 µm filters does not remove the particles formed by amiodarone HCl, and no loss in measured drug amount or total recovery is seen from HPLC is seen (table IX). Moving to 0.22 µm filters, most nanoparticles are removed (calculated to account for < 1 µM of drug from NTA, results not shown). A lower amount of drug measured in the filtrate by HPLC is seen, combined with lower recoveries, again suggesting some non-specific adsorption to the filter. However, the 0.22 µm filtrate response (containing no/few nanoparticles) is still higher than that obtained when starting from solid (table IX). This compound is demonstrating true supersaturation i.e. the samples appear to contain molecularly dissolved content. An interesting point to note is that a decrease in % recovery is only seen when the nanoparticles are removed, despite use of the same filter membrane. This suggests that the loss in recovery could potentially be due to nanoparticle adsorption rather than adsorption of the molecularly dissolved content.

4.3.3 Precipitation methods and varying incubation times

Intuitively, solubility values obtained by precipitation should depend on the incubation time employed. For all of the previous results, no incubation time was

employed – separation of the liquid and solid phases took place immediately after the aqueous phase was added to the DMSO stock. Both HPLC (with centrifugation) and NTA analysis (no separation) were employed to investigate the effect of time on the solubility results (table XI and XII).

Table XI: Amount of drug present in the supernatant after centrifugation for tolnaftate, clotrimazole and amiodarone HCl, as a function of time

	Amount of drug present (µM) in the supernatant after centrifugation (9,300 x g rcf, 5 mins)		
Incubation time (mins)	***Amiodarone HCl***	***Clotrimazole***	***Tolnaftate***
0	27.5 ± 1.5	20.2 ± 3.2	13.3 ± 5.6
10	23.8 ± 0.3	-	*9.4 ± 6.7*
60	22.9 ± 0.5	16.5 ± 0.5	-
180	-	18 ± 1	***3.0 ± 0.5***
1440	24.0 ± 0.3	***2.9 ± 0.2***	****5.1 ± 1.8***

**poor total recovery value obtained*

As expected, the solubility values obtained by HPLC for all 3 compounds decreases with time. The time taken to become close to the solubility from solid value however varies dramatically between compounds (values bold and italicised). Tolnaftate is very close to the solubility from solid value after only ten minutes incubation; clotrimazole is still well in excess of the solubility from solid value after 3 hours incubation, but has reached a similar value after 24 hour incubation; amiodarone HCl is still well in excess of the solubility from solid even after a 24 hour incubation period. What remains unknown is why clotrimazole, having a lower 'apparent' supersaturation than amiodarone HCl, reaches an equilibrium value faster. The NTA results in table XII add further insight;

Table XII: NTA analysis of sample suspensions for all three model compounds, incubated for various amounts of time at room temperature

		Average particle diameter (nm)		Average particle concentration (E^6 particles /mL)		Calculated nanomoles of drug present as nanoparticles	
Compound	***Initial rate of increase of mean diameter (nm/min)**	**t0**	**End of fast initial particle growth**	***t0***	***End of fast initial particle growth***	**t0**	**End of fast initial particle growth**
Ami	2	118 ± 3	270 ± 38	19.8 ± 2.5	6.4 ± 1	6 ± 1	27 ± 10
Clot	1	215 ± 3	450 ± 24	9.9 ± 2.8	0.5 ± 0.1	26 ± 8	>100 % of added
Tol	7	185 ± 19	635 ± 26	19.1 ± 3.0	2.8 ± 0.4	36 ± 16	>100 % of added

**initial rate of mean diameter increase taken as the initial slope in the graph of mean particle diameter against time; for tolnaftate and amiodarone HCl, the fast initial rate increase occurred between t0 and t 60. For clotrimazole it occurred between t 0 and t 180. Note this is a rough indication of the average particle diameter increase rate to give a comparable variable between compounds. Diameter and concentration results are for the start and end of this fast initial growth period.*

As detailed previously, the compounds can be ranked in terms of 'apparent' supersaturation (Tolnaftate>amiodarone>clotrimazole). In terms of the initial mean diameter increase rate seen, this ranking holds true. Having now looked at several methods of solubility behaviour determination, some overall conclusions for each compound and its individual precipitation behaviour can be deduced.

Tolnaftate has fairly straightforward precipitation behaviour. It 'immediately' forms nanoparticles upon mixing DMSO and buffer solutions. As expected from a system with high apparent supersaturation, it reaches equilibrium solubility rapidly. The enhanced HPLC response is merely due to the difficulties in completely removing the nanoparticles present – no molecularly dissolved drug above the solubility from solid value appears to be present. The 'supersaturation' seen is therefore not 'true'

supersaturation. Fast particle growth kinetics are seen; after 3 hours, the particles have grown to a large enough size to allow removal by centrifugation.

Clotrimazole has slightly more complicated precipitation behaviour. As with tolnaftate, it forms substantial numbers of nanoparticles upon mixing the aqueous and organic components together. In contrast to tolnaftate, these particles do not account for the full HPLC supernatant response (based on calculations). We have also shown (from filtrate HPLC response where filtrate is known from NTA to contain few particles) that there is dissolved drug content above the solubility from solid value. 'True' supersaturation is therefore seen. This is despite the fact that nanoparticles have already formed and thus initial nucleation has already begun. This means while initial nucleation is rapid, growth appears to be slow. Slow growth/precipitation to equilibrium is also seen from tables XI and XII. From table XI, clotrimazole precipitation does eventually 'finish' at the 24 hr time point i.e. the particles have grown to a size that can be centrifuged out and the amount of drug remaining in solution is close to the solubility from solid value.

Amiodarone HCl also forms nanoparticles as the first measurable step in the precipitation process. As with the other two compounds, precipitation methods with HPLC analysis give the highest solubility results. Particles are still present in the supernatant, although they are calculated to only contribute to a few µM. Amiodarone HCl therefore has behaviour similar to clotrimazole; despite the presence of nanoparticles, there is still a significant amount of drug present molecularly dissolved. It can be seen from table XII that amiodarone HCl initially forms very small (and weakly scattering relative to the other two compounds) particles. This is one of the main differences between this compound and the other two - amiodarone HCl particles are around half the size of those formed by tolnaftate and clotrimazole. Another difference is that they cannot be removed by 0.45 µm PVDF filters. This compound also unexpectedly, based on 'apparent' supersaturation, shows the highest HPLC supernatant amounts, both initially and after a 24 hour incubation period. This can now be linked to the small size of the

particles formed. Despite a slowing of the particle growth rate at t60, amiodarone HCl particles are still < 300 nm in diameter after an hour's incubation. The small size of the particles means that there is little change in the amount of compound measured in the supernatant over time by HPLC - despite growing at a reasonable rate, they do not reach a large enough size to be removed fully by the centrifugation employed and are counted in the HPLC response along with any molecularly dissolved content. It should also be noted that a similar time course experiment on the HPLC was performed with amiodarone HCl only, using 0.22 μm filters (results not shown). Even after 24 incubation, the filtrate (containing no/few nanoparticles) was still in excess of the solubility from solid value (12.0 ± 0.1 μM), meaning the compound was still supersaturated, despite having a large number of nanoparticulate nuclei present. A combination of amiodarone HCl's small initial particle size and the stability of the samples at concentrations above the equilibrium mean that it shows the slowest overall kinetics of the three compounds, despite having a high sample 'apparent' supersaturation.

The determination of the kinetic solubility, using HPLC, of all three compounds investigated here was complicated by the presence of nanoparticles. The centrifugation parameters employed were not sufficient to remove all of the particles; filtration however gave compound dependent results, and for one compound, the nanoparticles could pass through the filter membrane completely. Thus determining whether the samples were truly supersaturated or whether the HPLC response was partly due to the presence of nanoparticles involved several other experiments. Of course, should a compound form larger, micron-sized particles 'immediately' or at least on a fairly short time scale (like tolnaftate), these problems are largely avoided. Several papers have compared different methods of kinetic solubility determination for a large number of compounds;[3-8] crucial differences in the work performed here include the use of a light scattering method with a lower limit of detection, and a detailed investigation into the type of particles formed and their behaviour.

As mentioned previously, it is known, that a large number of compounds form 'aggregates' of a few hundred nm in size upon mixing DMSO stock solutions and aqueous buffers at the μM concentrations relevant here. [12, 15, 13, 14] This is well documented in bioassay and screening literature, but the impact of the sort of particles on the results of experiments similar to those performed during solubility screening has not been assessed until now. Here we show that the separation method used can affect the results of the solubility assay, with centrifugation potentially resulting in incomplete removal of nanoparticulate material, and filtration giving compound dependent results with regard to nanoparticle removal and adsorption. As expected, time has an effect on the samples –this change in response can be due to both supersaturation effects and growth rate of nanoparticles to larger, removable sizes. We also show that, while initial particle formation for all three compounds investigated here was rapid, growth was much slower.

4.4 Conclusions

Several conclusions can be drawn from the work presented. In terms of the analytical method used, determination of the 'kinetic solubility' by NTA using a precipitation method gives results closest to that obtained by solubility from solid. In terms of determining the solubility via precipitation using HPLC analysis, several factors can dramatically affects the results of the experiment. On a sample preparation level, the separation method used can change the results of the solubility assay due to the small particle sizes encountered here. Centrifugation can result in incomplete separation of the nanoparticles and the molecularly dissolved content, which can lead to an increase in kinetic solubility solely due to nanoparticle presence. The extent of this increase depends on the centrifugation parameters employed, the density of the particles and the size of the initial particles. Of course, the analysis of compounds which formed larger sized particles would not encounter these problems. Filtration using a 0.45 µm filter membrane can actually remove nanoparticles smaller than expected (~200 nm), likely due to adsorption. At smaller sizes (~100 nm), the particles can pass through the filter and a 0.22 µm filter is required. For all three compounds, low total % recovery was obtained using filters, suggesting some type of non-specific adsorption.

From the point of view of the precipitation process itself, fast nucleation and subsequently relatively slower growth to larger sizes is a common theme for all three compounds. As expected, incubation time decreases the kinetic solubility value obtained by HPLC. The rate at which this decreased occurred varied for all three compounds. This was rationalised by looking at the supersaturation of the sample, the initial nanoparticle growth rate, the initial size of the particles formed and the ability of the compound to demonstrate some supersaturation. Here we have used a limited selection of 3 structurally unrelated, poorly water soluble test compounds – further and more complete understanding of the process would involve similar experiments with a larger sample set.

4.5 References

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5. The effect of % DMSO on solubility measurements and nanoparticle behaviour

5.1 Introduction

The use of DMSO as a compound storage or stock medium is commonplace within the pharmaceutical industry. Aliquots of the DMSO stock solution are then utilised for various different analyses, such as high throughput screening (HTS) biological activity assays, and solubility screens at the discovery stage. These analyses both typically involve the addition of the DMSO stock to an aqueous buffer system - since most compounds are hydrophobic, there is the possibility of precipitation on the timescale of the analysis. Within a kinetic solubility setting, precipitation is an anticipated phenomenon, and is even used as a way of detecting when the solubility limit has been reached.[1] Within a bioassay setting, it is assumed precipitation does not occur, and the test compound is fully dissolved and able to interact with whatever target is being used (e.g. cell, enzyme). The precipitation of the compounds from the bioassay media is a recognised issue, and can often interfere with the results of the assay.[2] Both false negative (due low amount of drug dissolved and available to interact with target) and false positive (aggregates of the compound non-stoichiometrically inhibit the target) results could potentially be obtained.

Various controllable factors within the experimental set up for both types of assay could theoretically affect the precipitation process, which in turn would affect the results obtained. One of these factors is the percent DMSO present in the final sample composition. Within a bioassay setting, the amount of DMSO present in samples is kept to a minimum (maximum 5% by volume), in order to reduce any interference effects on the assay. This is the main concern with the DMSO present, rather than its effect on any compound precipitation. In kinetic solubility analyses, varying percentages of DMSO can be used depending on the protocol, although as

with bioassays, it is generally kept to a minimum. DMSO is reported as having both 'co-solvent' and 'supersaturation' effects on kinetic aqueous solubility values i.e. affects both the equilibrium solubility and the ability of the system to supersaturate. Bard *et al* [3] reported that enhanced kinetic solubility values were mainly due to a compound's ability to form stable supersaturated solutions when prepared from DMSO stocks, with co-solvent effects only being modest. Chen *et al* reported an increase in solubility when samples had DMSO present (starting from the stock solution, using a precipitation method) compared to those with no DMSO present (starting from the solid, using a 'shake-flask' type dissolution method). The group noted that poorly soluble compounds could be affected significantly.[4] Martel *et al* noted that increasing the percent DMSO resulted in solubility values which had less correlation to those obtained with no DMSO present. They also noted the effect was highly compound specific.[5] Here we explore in detail the effect of the % DMSO on the precipitation of 3 poorly water soluble test compounds (amiodarone HCl, clotrimazole and tolnaftate), at concentrations typically used in bioassay and kinetic solubility screening. Previously (chapter 4), the kinetic solubility of all three compounds was explored in detail at 1% DMSO by volume. Here, we increase this to 5% DMSO, while retaining the same total drug amount added, and compare the results of the experiments.

5.2 Materials and Methods

The sample preparation details, instrumental details, methods and materials are all as used for 1% samples in chapter 4. All that differed was the volumes of DMSO and buffer, and the concentration of the DMSO stock used. For example; to prepare a 50 µM (total volume 1 mL), 5% DMSO by volume suspension of drug, 50 µL of a 1 mM stock solution in DMSO was pipetted. 950 µL of the appropriate buffer was then added.

5.3 Results and Discussion

5.3.1 'Shake-Flask' solubility

The following results were obtained from the 'Shake flask' solubility experiments;

Table I: 'Shake-Flask' solubility results

	'Shake-Flask' solubility ± S.D (µM)		
Compound	**1% DMSO/Buffer**	**5% DMSO/Buffer**	**p value for t-test comparing 1 and 5% DMSO solubility values**
Amiodarone HCl	1.9 ± 0.2	3.7 ± 0.8	Sig (0.008)
Clotrimazole	4.3 ± 1.3	6.6 ± 1.5	*NS (>0.05)
Tolnaftate	1.5 ± 0.8	1.6 ± 0.8	*NS (>0.05)

Ami – n =15 (average of 5 time points, 3 samples per time point), Clot – n=9 (average of 3 time points, 3 samples per time point), Tol – n=15 (average of 5 time points, 3 samples per time point).**Where NS = not significant, 95% confidence interval used*

As can be seen from Table I, all three compounds show a slight increase in going from 1 and 5% DSMO/buffer mixtures. However, only amiodarone HCl samples show any statistically significant enhancement from a 5% DMSO/buffer mixture compared to a 1% DMSO mixture, and in terms of solubility, both 1 and 5% DMSO results would still be thought of as poorly soluble. Thus while a co-solvent effect is present for these samples, it is fairly modest. However, amiodarone HCl precipitation samples containing 1 and 5% DMSO will, for the same amount of drug added, have slightly different 'apparent' supersaturations (total amount of drug added/'shake-flask'solubility value) – this will be borne in mind in later discussions. Both tolnaftate and clotrimazole have very similar 'apparent' supersaturations, for the same total amount of drug added, regardless of the % DMSO present.

5.3.2 Kinetic Solubility Assay Results

(i) Precipitation method 1 – NTA based

As with Chapter 4, 2 methods were used to determine the kinetic solubility of the 3 compounds. Method 1 involved using NTA (light scattering) to determine the compound concentration at which the detected nanoparticle concentration becomes significantly different from a blank sample, as described in chapter 4. The results obtained for the 1% DMSO samples are documented in that chapter; here the equivalent 5% DMSO samples were tested using the same settings as a comparison (Table II).

Table II: Kinetic solubility determination by NTA

	Drug concentration at which particle formation occurs	
Compound	***1% v/v DMSO***	***5% v/v DMSO***
Amiodarone HCl	5 µM	4 µM
Clotrimazole	16 µM	16 µM
Tolnaftate	2 µM	2 µM

The results from both 1 and 5% DMSO are very similar, regardless of the compound investigated. As discussed in chapter 4, these are also fairly similar to the solubility from solid values obtained. This suggests no effect of the % DMSO present on the concentration at which nanoparticle formation is initiated. Initial nucleation at concentrations fairly close to the equilibrium solubility is therefore not affected.

(ii) Precipitation method 2 – HPLC based

In this method, an excess of compound is added via the aliquot of DMSO stock solution such that the total drug amount is greater than the solubility from solid amount. Separation of the precipitate from the molecularly dissolved compound is then performed. Shown are the results of HPLC analysis where either centrifugation or filtration has been used as the solid/liquid separation method (table III).

Table III: Comparison of results obtained from samples containing 1 and 5% DMSO, using precipitation method 2, analysed by HPLC after either centrifugation or filtration

	Centrifugation (5 mins, 9,300 x g rcf)	**0.45 µm PVDF filtration**	**0.22 µm PVDF filtration**
Compound	***5% DMSO supernatant value as a % of 1% DMSO supernatant value***	***5% DMSO supernatant value as a % of 1% DMSO supernatant value***	***5% DMSO supernatant value as a % of 1% DMSO supernatant value***
Amiodarone HCl	145%	140%	213%
Clotrimazole	154%	232%	Not performed
Tolnaftate	235%	*N/A	Not performed

**1% DMSO samples showed no peak after filtration, 5% DMSO samples showed a small peak, but inconsistently.*

Note that in-depth discussion of causes of variance between separation methods has already been performed in chapter 4, and will not be repeated here. Only the results of 1 and 5% DMSO samples for any given method will be compared. All of the results obtained for 1% DMSO samples are also given in chapter 4; where possible, ratios or percentages will be presented to avoid repetition. 5% absolute values are available in the appendix.

All compounds show significantly higher 5% values compared to 1% values, regardless of the separation method used. Standard deviations for each sample set

are small, and experimental error does not account for the difference (full results with standard deviations are detailed in the appendix). As mentioned in chapter 4, all 1% results are in excess of the solubility from solid values. Since the results in section 5.3.1 showed no thermodynamic increase in solubility for tolnaftate and clotrimazole when moving to a 5% DMSO solvent system, the effect must be kinetic. For samples where centrifugation was employed as the solid/liquid separation method, tolnaftate shows the largest difference between 1 and 5% samples. The samples containing 5% DMSO have concentrations of tolnaftate measured in the supernatant that are more than double that measured from 1% DMSO samples. Filtration results for this drug with a 5% sample composition were irreproducible; sometimes a small peak was detected, sometimes not.

Investigating the nanoparticle/liquid separation method employed for the 5% DMSO samples using NTA gives the following results (Table IV).

Table IV: NTA results of samples containing 5% DMSO, after employment of centrifugation or filtration

	5 mins 9,300 x g cent			**0.45 um PVDF filter**		
Compound	**Diameter (nm)**	**Particle concentration (10^6 particles/mL)**	**Calculated nanomoles of drug**	**Diameter (nm)**	**Particle concentration (10^6 particles/mL)**	**Calculated nanomoles of drug**
Amiodarone HCl	116 ± 3	22 ± 1	6 ± 1	110 ± 3	23 ± 8	7 ± 3
Clotrimazole	212 ± 14	6 ± 2	14 ± 8	< 100 tracks*		
Tolnaftate	197 ± 18	9 ± 7	15 ± 9	particles detected inconsistently, sometimes <100 tracks*		

**<100 tracks – low track numbers mean inaccurate mean diameter and very low particle concentrations*

From Table IV, we can see that, as for the 1% DMSO samples, centrifugation does not remove all of the particles formed. Filtration has more success in performing this, and, as for 1% DMSO samples, amiodarone HCl requires the use of a 0.22 µM PVDF filter for removal of the majority of particles. Whilst similar trends are therefore seen between the 1 and 5% DMSO samples, what should be highlighted are the calculated nanomoles of drug present in the form of nanoparticles left after centrifugation (Table V);

Table V: Calculated µmoles of drug per L suspension in the form of nanoparticles after centrifugation for 1 and 5% DMSO samples, based on NTA data

	Calculated µM of drug after centrifugation	
Compound	**1% DMSO**	**5% DMSO**
Amiodarone HCl	3 ± 0.3 (n=3)	6 ± 1 (n=3)
Clotrimazole	unable to quantify (n=4)	14 ± 8 (n=4)
Tolnaftate	14 ± 6 (n=6)	15 ± 9 (n=4)

Tolnaftate 1 and 5% DMSO samples show similar amounts of drug in remaining in the supernatant after centrifugation in the form of nanoparticles from NTA, but different supernatant drug concentrations after HPLC analysis. The 5% DMSO samples had increased drug amounts detected in the supernatant by HPLC compared to the 1% DMSO samples – the NTA results shown in table V suggest that this cannot be attributed to increased concentrations of drug present as nanoparticles. No 'true' supersaturation was seen for tolnaftate 1% DMSO samples (chapter 4) but here the results suggest some 'true' supersaturation in the 5% DMSO samples. Amiodarone HCl and clotrimazole 5% DMSO samples have increased calculated nanomoles of drug present as nanoparticles in the supernatant after centrifugation compared to 1% DMSO samples.

The use of filters (0.45 or 0.22 µm PVDF, depending on the compound) has been shown by NTA to completely remove the drug nanoparticles, but HPLC analysis still shows an increase in the concentration of drug measured in the filtrate drug amiodarone HCl and clotrimazole 5% DMSO samples, again leading to the conclusion that 5% DMSO samples have an increased ability to remain supersaturated compared to 1% samples. This effect is in addition to having differences in the amount of particles formed. For tolnaftate, 5% DMSO HPLC filtration results were sporadic; sometimes a peak was detected in the filtrate, sometimes not. This was also reflected in the NTA results, where a fairly large range of nanoparticle concentrations were seen.

5.3.3 1 and 5% DMSO and incubation times

Since it seemed likely that the effect of 5% DMSO was one of a kinetic nature, experiments involving various incubation times were performed. Both HPLC and NTA were employed to analyse changes in the samples as incubation times were varied.

Table VI shows the difference in measured HPLC supernatant amounts for 1 and 5% DMSO samples after 24 hours incubation. These are presented as a % of the solubility from solid value in Table I. As before, full details of the 1% incubation results are available in chapter 4; full details for 5% results are available in the appendix.

Table VI: 1 and 5% DMSO samples with varying incubation times, precipitation method 2 followed by centrifugation and HPLC analysis of the supernatant

	Kinetic solubility after 24 hr as % of 'shake-flask' solubility		
	Amiodarone HCl	**Clotrimazole**	**Tolnaftate**
1%	1263%	Close to equil.	Close to equil.
5%	1000%	374%	1088%

As can be seen from the table, all of the 5% DMSO samples are still well in excess of the 'shake-flask' solubility value, even after 24 hour incubation. Two of the three 1% DMSO samples have reached the equilibrium value – the difference in kinetics between the three compounds has already been discussed in the previous chapter. What is key to note here is the difference between the 1 and 5% DMSO samples. For amiodarone HCl, whilst the percentage detailed in table VI is slightly higher for the 1% DMSO sample, the absolute value for the 5% sample is higher (see appendix for full details of 5% DMSO samples). This is due to the slight difference in 'shake-flask' solubility values between amiodarone samples containing 1 and 5% DMSO

solvent systems. All 5% samples show increased drug concentrations present in the supernatant by HPLC analysis compared to the 1% DMSO samples, even after 24 hours. Although the 'shake-flask' solubility experiments performed for these compounds showed that the % DMSO does not cause any thermodynamic increase in solubility for 2 of the three compounds (and only a modest increase for the 3rd), 5% DMSO precipitation method 2 samples still show increased concentrations of drug detected by HPLC in the supernatant after 24 hours. This suggests the higher % DMSO slows the kinetics of the precipitation down dramatically. Investigating this further via NTA (Table VII);

Table VII: NTA, comparison of 1 and 5% DMSO results showing evidence of slower particle formation kinetics for 5% DMSO samples

	***Initial rate of increase in mean nanoparticle diameter (nm/min)**		**Mean particle diameter at the end of fast initial particle growth period* (nm)**		**Initial particle concentration (10^6 particles/mL) (no centrifugation)**		**Initial calculated nanomoles (no centrifugation)**	
Compound	**1% DMSO**	**5% DMSO**	***1% DMSO***	***5% DMSO***	**1% DMSO**	**5% DMSO**	**1% DMSO**	**5% DMSO**
Amiodarone HCl	2	2	*270 ± 38*	*237 ± 8*	19.8 ± 2.5	9.3 ± 1.9	6 ± 1	2 ± 0
Clotrimazole	1	0.3	*450 ± 24*	*278 ± 8*	9.9 ± 2.8	5.2 ± 1.4	26 ± 8	18 ± 4
Tolnaftate	7	2	*635 ± 26*	*307 ± 18*	19.1 ± 3.0	7.4 ± 4.0	36 ± 16	15 ± 6

** initial rate of mean diameter increase taken as the initial slope in the graph of mean particle diameter against time; for tolnaftate and amiodarone HCl, the fast initial rate increase occurred between t0 and t 60. For clotrimazole it occurred between t 0 and t 180. Note this is a rough indication of the average particle diameter increase rate to give a comparable variable between compounds. Mean particle diameter results are for the end of this fast initial growth period,*

indicating differences in growth over time. Particle concentration results are those obtained initially, and highlight immediate differences between the samples.

For all three compounds investigated, precipitation from a 5% DMSO sample system results in;

1. Lower initial particle concentrations
2. Lower calculated nanomoles of drug present as nanoparticles (initially)
3. Smaller particle diameters at the end of the rapid particle diameter growth period

compared to precipitation from a 1% DMSO sample system at the same total amount of drug added. For two of the three compounds, the initial rate of nanoparticle mean diameter increase (Table VII, column one) is lower for the 5% DMSO samples. Clotrimazole shows a slight lowering; the effect for tolnaftate is dramatic due to the rapid rate at which the 1% nanoparticles grow. This is also highlighted in the second column of table VII. The % DMSO present in the samples therefore has the greatest effect on the growth of tolnaftate nanoparticles. The rate of mean diameter increase for amiodarone HCl samples is unaffected by the percentage of DMSO present in the precipitation experiment – the difference in initial supersaturation could be a reason for this. NTA measured nanoparticle concentrations are affected by the % DMSO present; all 5% samples show lower initial particle concentrations. All of the initial calculated nanomoles are lower for 5% samples. Less drug is therefore present as particles at time 0 for 5%, meaning more is present in the molecularly dissolved solution. For amiodarone HCl and clotrimazole, some supersaturation is seen from 1% samples – increasing the DMSO present is therefore enhancing this effect. For tolnaftate, very little true supersaturation is seen for 1% samples – the drug appears to precipitate to equilibrium solubility almost immediately, as discussed in chapter 4. Here, however, the 5% samples show supersaturation, thus having a dramatic effect on the behaviour of the compound.

Looking at trends in particle concentrations over time, all 1% DMSO samples show a steady decrease at the measured time points. 5% DMSO samples, however, show an initial increase then decrease for all three compounds (data not shown). This again leads to the conclusion that 5% DMSO slows the precipitation kinetics of all three compounds – compared to the 1% DMSO samples, more time is taken for the maximum particle concentration to be reached.

5.4 Conclusions

Increasing the % DMSO present in the final sample composition has, for these three compounds, varying results depending on the method used to assess solubility. In terms of solubility from solid, only one compound showed any statistically significant enhancement with the increased percentage of DMSO present. This increase was also fairly modest, around a factor of 2, but with both values remaining below 5 µM. So with this method, overall 1 and 5% samples were similar. Using the NTA to determine the concentration at which precipitation began also yielded similar results from 1 and 5% samples. This method of course uses various concentrations which are fairly close to the solubility from solid value – it was not necessary to add in a large excess of compound in order for nanoparticle formation to be initiated (for these three compounds). Moving to the precipitation-HPLC based method, a large excess of compound is added, so that the amount of drug present in the system is way above that obtained from the solubility from solid experiments. It would therefore be expected that rapid precipitation to equilibrium would occur. This is not the case – whilst initial nanoparticle formation is rapid, the samples still have dissolved drug in excess of the equilibrium solubility value. Thus, subsequent growth/ripening/ further formation of the particle is the slow, rate determining step, rather than initial nucleation. In samples containing 5% DMSO, this effect is exacerbated - nanoparticle formation and growth is slowed. Although particles are present from time 0, there are fewer compared to the 1% DMSO samples. Samples with 5% DMSO are therefore supersaturated for longer compared to their counterpart 1% samples.

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6. Non-Stoichiometric, aggregate based inhibition: Bioassay trends explained via nanoparticle growth measurements

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Keywords – Non-stoichiometric aggregate based inhibition, bioassays, HTS, particle growth

6.1 Abstract

Non-stoichiometric, aggregate based inhibition has been a much studied phenomenon in recent years. Most of the work has focused on enzyme inhibition by the aggregator/nanoparticle former. Here, we look at the effects of varying amounts of dissolved protein on the nanoparticle precipitation of 3 model compounds (amiodarone HCl, clotrimazole and tolnaftate). The work demonstrates how protein content changes both the initial precipitation and further growth kinetics of the system. Using tolnaftate, we also show that the enzyme-nanoparticle interaction (hypothesized to be a surface adsorption type interaction) occurs to a greater extent when the protein is present whilst the particles are forming. These results are of importance to HTS and interpretation of discovery bioassay results.

6.1 Introduction

Recently, several publications have explored the phenomenon of non-stoichiometric, aggregate based inhibition as a source of false positive results in high throughput bioassay screening. [1-5] The aggregates interact with the target in a non-specific, non-stoichiometric manner, resulting in inhibition despite the lack of specific, drug like interaction.[2] Whilst in depth analysis of how these aggregates can affect enzyme inhibition and the results of activity screens has been performed already,[1-7] little is known about how the aggregates themselves behave under test conditions.[8] Here, we demonstrate that the dissolved protein content present in the assay medium significantly hinders the nanoparticle growth kinetics of 3 model compounds, amiodarone HCl (ami), clotrimazole (clot) and tolnaftate (tol). All drugs have low aqueous solubility (<10 μM), and form particles in the nanometre size range upon mixing DMSO stock solutions of the drugs with aqueous buffers. Clotrimazole has been shown previously to be a non-stoichiometric, aggregate based inhibitor.[2] Whilst the inhibition effects of tolnaftate and amiodarone HCl are unknown, they form particles within the size range typically seen for non-stoichiometric, aggregate based inhibitors. Over time, clear differences were seen between samples precipitated in the presence of dissolved protein, and those precipitated in buffer only. Using tolnaftate, it was also found that addition of enzyme to a nanoparticle suspension after precipitation had already been initiated by an initial aliquot of buffer alone resulted in no difference in kinetics of particle growth. This suggests the enzyme molecules must be present during the aggregate/nanoparticle formation process in order to adsorb to the nanoparticle surface. All of the factors studied here are likely to be variable in HTS bioassay protocols. The results are therefore directly relevant to this area of drug discovery.

6.2 Experimental

6.2.1 Material s and Methods

Amiodarone HCl (A-8423), clotrimazole (C-6019) and tolnaftate (T-6638) were purchased from Sigma-Aldrich. Compound purity confirmed by NMR, and either matched or exceeded the manufacturer's specifications. (≥ 98%).

DMSO used was UV-Vis grade, purchased from Fluka.

KH_2PO_4 and $Na_2HPO_4.2H_2O$ were purchased from Fisher Scientific.

Citric Acid was purchased from Fisher Scientific.

α-chymotrypsin (type II, bovine pancreatic, lyophilised) was purchased from Sigma (C-4129), protein content ≥ 85% (value obtained from supplier).

6.2.2 Stock and Buffer preparations

A 5 mM weighed stock of either amiodarone, clotrimazole or tolnaftate in 10 mL DMSO was prepared.

A pH 7.0 (± 0.05) phosphate buffer was prepared, using 0.0025 moles of $KHPO_4$ and 0.0025 moles of Na_2HPO_4 per litre of deionised, Milli-Q water. The pH adjustment was conducted with 2 M NaOH if required. The buffer was filtered using a 0.2 µm hydrophilic PTFE filter (Millipore), and then used to prepared a 1 mg/mL solution of α-chymotrypsin. Aliquots of this solution were then diluted to give 0.5, 0.1, 0.05 and 0.01 mg/mL solutions of α-chymotrypsin in filtered pH 7 buffer.

A pH 4.0 citrate-phosphate buffer was also used for amiodarone HCl samples. This was prepared by mixing 147 mL of a 0.1 M citric acid solution and 103 mL of 0.2 M sodium diphosphate solution and diluting to 500 mL with Milli-Q water. The buffer was adjusted to pH 4.0 (±0.05) using citric acid. The buffer was filtered and then used to prepared a 1 mg/mL solution of α-chymotrypsin. This was then diluted to give a 0.1 mg/mL solution of α-chymotrypsin in pH 4 filtered buffer.

6.2.3 Sample preparation

i. Compound precipitation initiated using buffer containing dissolved protein

10 µL of the 5 mM DMSO stock solution was pipetted into an HPLC vial. 990 µL of filtered pH 7 buffer containing various concentrations (0 to 1 mg/mL) of α-chymotrypsin was added to the vial. This gave a total of 50 nanomoles of drug in 1 mL of DMSO/buffer/protein mixture, with 1% DMSO by total volume. No further mixing was performed. The resulting suspension was transferred to the NanoSight chamber, either immediately, or after a 1 hour incubation period. Both precipitation and incubation steps were performed at 25 °C. For each time point and each α-chymotrypsin content, 3 replicate samples were prepared and analysed.

ii. Compound precipitation initiated using buffer only, with secondary dilution to introduce dissolved protein

10 µL of the tolnaftate 5 mM DMSO stock solution was pipetted into an HPLC vial. 990 µL of filtered pH 7 buffer was added to the vial, giving 50 nanomoles of tolnaftate per mL of suspension. After twenty seconds, the suspension was diluted using 0.5 mL of filtered pH 7 buffer, containing either no protein or 1 mg/mL protein, to give a total sample volume of 1.5 mL. After dilution, the total drug content was 33 nanomoles per mL and the total protein content was either 0 mg/mL or 0.33 mg/mL. After dilution, the suspensions were analysed using the NanoSight either immediately, or after one hour incubation. As before, precipitation and incubation were performed at 25 °C. For each time point and each α-chymotrypsin content, samples were prepared in triplicate.

6.3.4 Instrument Details

The NanoSight LM10 with NTA software version 2.1 was used in all experiments.

Video recording settings - Fixed camera settings were employed for each compound (table 1), to allow fair comparison of particle concentration estimate between samples. The NanoSight chamber was set to 25 °C. 90 second videos were recorded for all samples.

Table I: NanoSight camera settings used

Compound	Shutter	Gain
Amiodarone HCl pH 7	100	0
Amiodarone HCl pH 4	1500	500
Clotrimazole	100	0
Tolnaftate	66	0

Processing settings – All processing settings were set to automatic, with blur increased to '5x5'. During processing, 'Ctrl +0' were pressed to obtain a non-smoothed particle size distribution.

Blank -A blank of 1% DMSO and 99% buffer containing 1 mg/mL chymotrypsin showed no detectable particles at the camera settings used for sample analysis.

Data handling –Number mean diameters and estimate of particle concentration obtained for three replicate samples were averaged and standard deviations obtained. The particle size distributions were binned (20 nm), and the distributions of 3 replicate samples averaged. If error bars are shown, they represent the standard deviation of the 3 replicate samples.

6.3 Results and Discussion

Table II shows particle sizes found immediately after addition of the aqueous buffer to the DMSO stock, and after 60 min further incubation.

Table II: Average particle diameter for samples containing 0 and 0.1mg/mL α-chymotrypsin

	Number Mean Diameter (nm) average of 3 reps ± S.D			
Compound	***0 mg/mL protein, t 0***	***0 mg/mL protein t60***	***0.1 mg/mL protein, t0***	***0.1 mg/mL protein, t60***
Ami	158 ± 26	524 ± 32	168 ± 33	210 ± 23.9
Clot	190 ± 4	367 ± 3	221 ± 15	227 ± 4
Tol	186 ± 19	635 ± 26	200 ± 12	225 ± 17

From Table II, regardless of the amount of protein added, the particles formed at time zero are very similar in diameter. However, the particles formed by all three compounds grow substantially over the incubation period in the absence of protein, but in the presence of 0.1mg/mL protein, growth is inhibited and only very increases in diameter are obtained. Table III shows the results of NTA concentration analysis.

Table III: Average particle concentrations for samples containing 0 and 0.1 mg/mL protein

	Particle concentration (10^8 particles/mL) average of 3 reps ± S.D.			
Compound	**0 mg/mL protein, t0**	**0 mg /mL protein, t60**	**0.1 mg/mL protein, t0**	**0.1 mg/mL protein, t60**
Ami	25 ± 4	5 ± 0.1	10 ± 8	15 ± 1
Clot	14 ± 2	2 ± 1	1 ± 0.4	7 ± 1
Tol	19 ± 3	3 ± 0.4	7 ± 2	18 ± 4

From table III, the particles formed by all three compounds grow substantially over the incubation period in the absence of protein, but in the presence of 0.1mg/mL protein, growth is inhibited. Samples containing no protein show a decrease in concentration over time as well as growth, whereas samples containing protein show little to no growth over time and an increase in particle concentration from time 0 to 60 mins (particle concentration data in appendix). A potential explanation is that the protein 'forces' the compound to form more particles in order to 'complete' the precipitation process. This would allow the samples to tend towards an equilibrium solubility value by forming more particles, rather undergoing ripening/growth of the particles already formed (as seems to be the case without protein present). This correlates well with the enzyme molecules adsorbing to the surface and in essence 'blocking' particle growth/ripening. An interesting effect was noted with amiodarone HCl – at pH4, no effect on particle concentration or size was seen from the presence of 0.1 mg/mL protein (data available in appendix). A possible explanation is that the compound, being a weak base, is more positively charged at pH 4 compared to 7 (pKa 10.2[9]) and thus has even greater repulsion towards the also positively charged chymotrypsin (pI 8.67). However, even at pH 7 the molecule would carry a substantial charge, and this may only partially explain the effect.

In order to explore the effect of enzyme concentration on precipitation, tolnaftate was precipitated in the presence of varying concentrations of protein (table IV which shows average particle diameter results, figure 1 which plots particle concentration results and figure 2 which shows particle size distribution results.).

Table IV: Tolnaftate and varying concentrations of chymoptrypsin, average particle diameters

	Number mean diameter (nm) average of 3 reps ± S.D.	
Alpha-chymotrypsin concentration, mg/mL	***time 0***	***time 60 min***
0	186 ± 19	635 ± 26
0.01	186 ± 9	264 ± 7
0.05	185 ± 6	259 ± 5
0.1	200 ± 12	225 ± 17
0.5	191 ± 13	237 ± 3
1	195 ± 6	199 ± 31

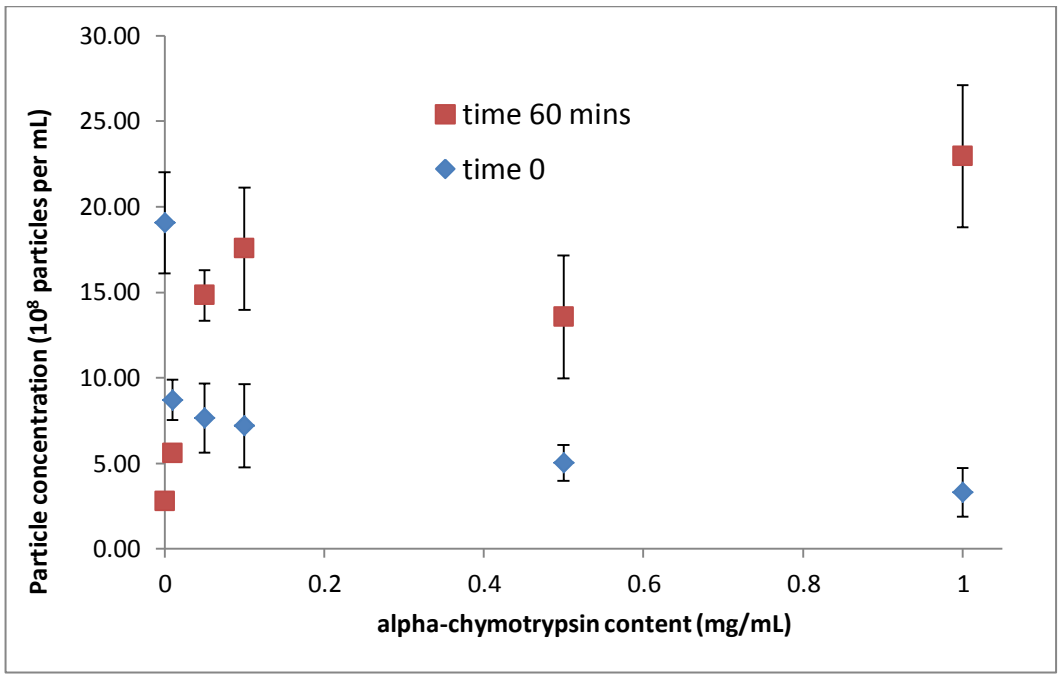

Figure 1: Tolnaftate - particle concentration Vs protein content at time zero and time 60 mins

Even as little as 0.01 mg/mL present results in samples which differ dramatically compared to 0 mg/mL protein. It can also be seen from the concentration results that, in general, as the protein content is increased (whilst maintaining the same amount of drug), the effect on the particle concentration formed changes. 0.01 and 1mg/mL show significantly different behaviours, both immediately and after a 60 minute incubation. Further investigation into the effect of protein concentrations below 0.01 mg/mL could be of use, and could determine the minimum amount of protein required for an effect.

The amount of drug present in particles can be estimated from their concentrations and sizes:[10] this indicates that at the highest protein concentration, only around 13% is particulate initially, but nearly all after 60 min. It is clear that all of the protein concentrations explored here slows both initial nanoparticle formation, and subsequent growth.

At time 0, all samples show monodisperse particle size distributions (see appendix) Figure 2 shows the particle size distributions obtained after 60 min.

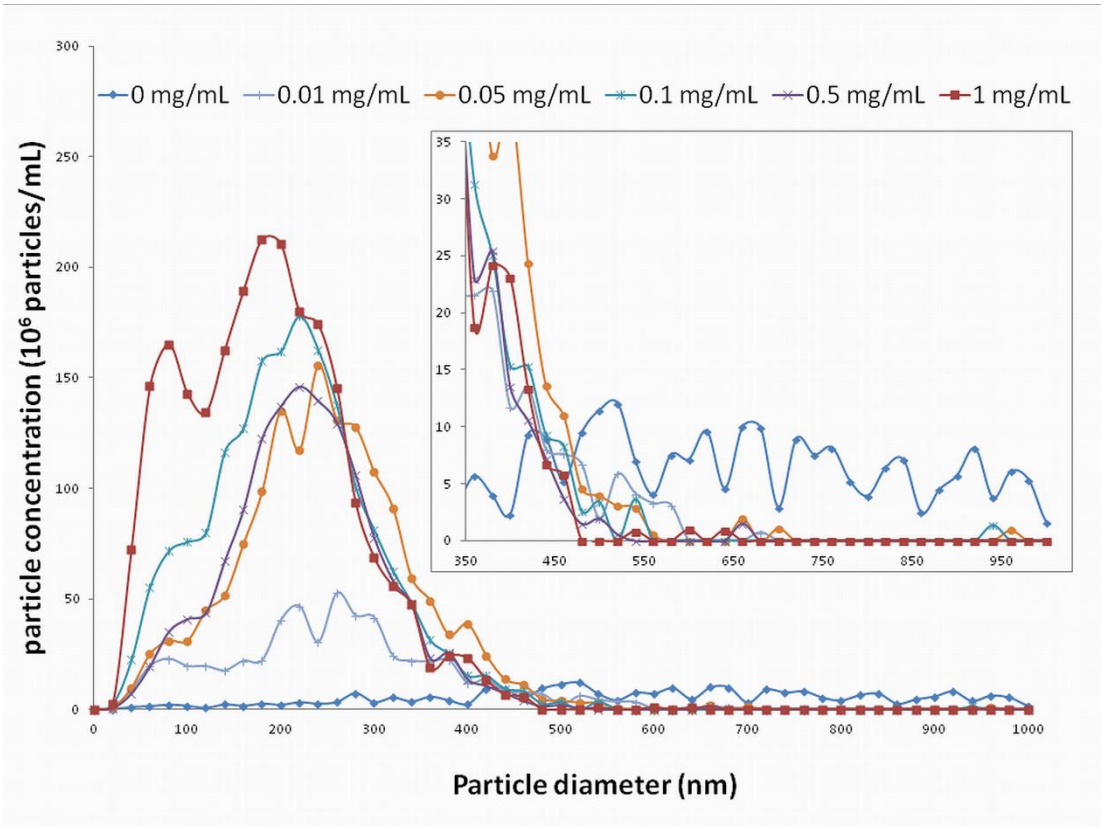

Figure 2: Average particle size distributions for tolnaftate samples containing various protein amounts, time 60 mins, with insert of close up 350-950 nm section.

Samples with 1 mg/mL α-chymotrypsin present have remained fairly monodisperse, with little ripening or growth seen, in stark contrast to those with no protein present. Higher concentrations of protein induce the formation of a population of smaller particles (~100 nm) after 60 minutes– these are not present at time 0 and likely form after the initial nanoparticle precipitation.

All 3 compounds explored here are form nanoparticles in the size range typically seen from non-stoichiometric, aggregation-based inhibitors. The effects seen here with regards to the presence of protein during precipitation initiation help explain some of the trends seen in previous inhibition studies. Time dependent inhibition was one of the distinguishing behaviours. [1-3] IC_{50} values typically decreased (improved) when the inhibitor and enzyme were pre-incubated for five minutes. As the formation of nanoparticles is a kinetic process, there is likely a change in particle concentration/size between zero and five minutes. If more particles are formed

after 5 minutes compared to at time 0, more surface is available for enzyme attachment, increasing non-stoichiometric inhibition and decreasing IC_{50}. Enzyme concentration is also known to influence non-stoichiometric inhibition. [1-3, 11] A ten-fold increase in enzyme concentration was observed to significantly increase (worsen) the IC_{50} value, and such an effect has been suggested as a means of detecting non-stoichiometric inhibition.[11] From the results presented earlier, an increase in protein concentration inhibits initial particle formation, so there are less particles available to interact with and inhibit the enzyme. Note however that particle formation is not prevented completely but merely slowed – so the extent of inhibition may be time and compound dependent. Similar effects may help explain the observation that excess protein (0.1 mg/mL BSA,[1] lysozyme and trypsin[7]) decreased inhibition by the nanoparticles. Finally, demonstration of the effects of protein on the nanoparticles is entirely consistent with the view that protein molecules interact with the particle surfaces. Note this interaction has some features in common with protein coated microcrystals (PCMCs). PCMC formation is a method of enzyme immobilization by which proteins are coated onto the surface of a micron-sized 'carrier' crystal during crystal formation.[12] Coan *et al* have previously shown that aggregates are not disrupted by high protein concentrations in the same way as they are with surfactants. The results shown here are consistent with this - even at protein concentrations as high as 1 mg/mL, nanoparticles are still present.

With the aim of further investigating the mechanism of the effects, protein was added to the system after precipitation had been initiated. Buffer was added to the DMSO stock solution of tolnaftate as before, initiating the precipitation process, but after 20 s, an additional 0.5 mL of filtered pH 7 buffer was added, containing either 0 or 1 mg/mL protein. Dilution alone reduces the subsequent growth of the nanoparticles (table V).

Table V: Diameter and concentration results, protein added after precipitation of tolnaftate

	Number mean diameter (nm - average of 3 reps ± S.D.)		***Particle Concentration (10^8 particles/mL - average of 3 reps ± S.D.)***	
Sample diluted with;	**Time 0**	**Time 60**	**Time 0**	**Time 60**
Buffer only	206 ± 16	297 ± 43	5.0 ± 0.8	10.0 ± 2.8
1 mg/mL chymotrypsin	204 ± 10	260 ± 11	3.4 ± 1.6	10.8 ± 0.8

Dilution had a large effect on particle growth for the control (no protein) samples (table V), and has slowed the process. Both the total amount of drug added and the incubation volume have changed here compared to previous samples**.** However, now there is little effect of protein, either immediately after its addition or upon further incubation. It could be expected that this would slow the process even further if the samples behaved as described previously. The data here, however, shows no synergistic or even additive effect– samples diluted with buffer containing protein now behave exactly as the control samples do. The particle size distributions confirm the similar behaviour.

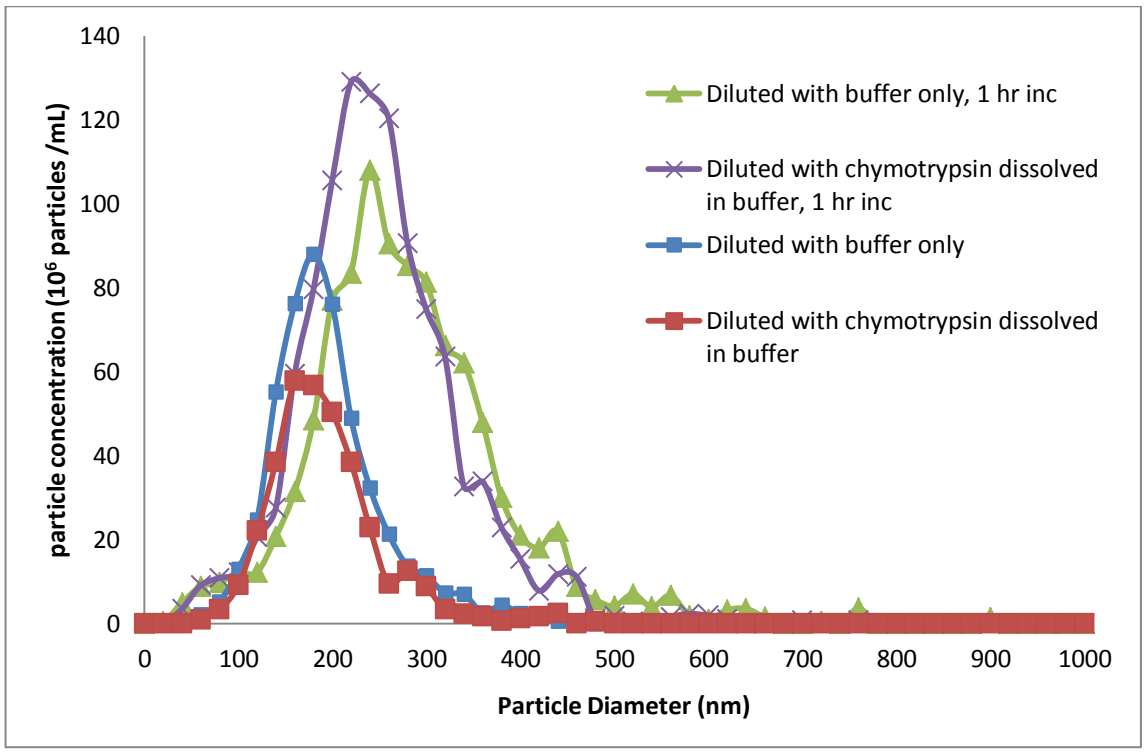

Figure 3: Particle size distributions, protein added after precipitation

The findings could also be of immediate practical relevance to screening. It should be relatively easy to adopt a two-stage mixing procedure i.e. addition of an initial aqueous portion (free from protein) to the DMSO stock, to begin compound precipitation, then subsequent addition of a further aqueous portion containing the enzyme, to begin the activity assessment. This would allow initial precipitation to occur before the enzyme is present. Of course, in an ideal world compound precipitation would not occur, but realistically it is a likely event for a large number of screening compounds due to their hydrophobicity. This approach, however, could potentially reduce the interaction of the enzyme and aggregate, thus reducing the number of false positive results, with only minor changes to the dilution procedure. Obviously more work is needed, but this idea may warrant some further investigation by those involved in bioassay screening.

6.4 Conclusions

In conclusion, we show that protein significantly affects the precipitation kinetics of 3 model compounds, with 0.1 mg/mL of α-chymotrypsin slowing both formation and ripening of drug nanoparticles. As little as 0.01 mg/mL has an effect. The slowing effect of protein on compound nanoparticle precipitation kinetics may explain some effects noted in studies of non-stoichiometric aggregate-based enzyme inhibition. These effects (sensitivity to enzyme concentration and time dependent inhibition) correlate well with the results seen here, in that the size and concentration of compound nanoparticles formed are dependent on both the amount of protein present and time. Interactions appear reduced if protein is added after precipitation is initiated, so this might be a valuable strategy in screening, although dilution alone alters the kinetics as well. Further investigation to see if the results obtained here translate into a practical recommendation would be of use. Better understanding of non-stoichiometric inhibition requires study of effects on particle precipitation itself, and extension of this work to other compounds and proteins.

ACKNOWLEDGMENTS

With thanks to MSD and University of Strathclyde for funding contributions

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7. Attempts at nanoparticulate crystalline form determination

7.1 Introduction

The crystalline form of a substance is directly related to physical properties such as solubility, melting point and dissolution rate.[1] Knowledge of the crystal form of a compound is therefore very important within the pharmaceutical industry.

Solids can exist in a variety of different crystalline forms – these are known as polymorphs, and differ in the arrangement of molecules within the crystal lattice. Solids can also be amorphous in character, show no long range order in the molecular arrangement within the solid, and thus do not exhibit Bragg diffraction. Compared to the most stable crystalline state, amorphous materials are a higher energy solid form, and tend to be more soluble than crystalline material.[2] Of course, as amorphous material is not the most stable solid form of the drug, conversion to the crystalline form occurs over time. This is one of the difficulties in fully assessing the solubility advantage for amorphous solids. [2]

With regards to compound precipitation from mixing DMSO stock with aqueous buffers, little published work is available on the solid form of the precipitate formed. Hoelke *et al*[3] used x-ray diffraction (XRD) to analyse the solid formed after precipitation from DMSO and buffer mixtures. Both model compounds investigated precipitated in an amorphous form. They conclude that the addition of DMSO stock solutions to aqueous buffers results in conditions which are too harsh for crystallisation, and thus fast precipitation of amorphous solid occurs. However, Sugano et al used polarised light microscopy (PLM) to investigate the crystallinity of 26 structurally diverse, low solubility drug molecules after precipitation from DMSO and buffer solutions. They found that 58% of their test compounds precipitated as crystalline after a 10 minute incubation period, and that this increased to 73% after 20 hours incubation. The compounds which precipitated as amorphous solids had solubility values in excess of those obtained from the solid. The technique (PLM)

was fully automated and so useful in solubility screening. The total drug concentration used here was however, fairly high in terms of screening (0.3 mM).[4]

Both theory and some limited results from the literature show that the solid form of the precipitate can affect the amount of drug left dissolved in solution. This would therefore affect the results of both an HTS activity screen and kinetic solubility experiments. All three of the drug molecules investigated in this thesis form nanoparticles upon precipitation from DMSO and buffer solutions. These particles then grow and ripen over time. This chapter documents one of the most challenging aspects of this work – attempts at establishing the crystalline form of the nanoparticulate precipitate. In order to do so, one compound (tolnaftate) was picked to trial various approaches. Due to the nature of the samples, the low concentrations used here (μM) and the small size of the initial precipitate, analysis was not straightforward. Several different techniques and approaches were considered, with only a few modest successes. Raman analysis of tolnaftate gave no conclusive results with regards to crystallinity, however could obtain a signal from a 1 mL scale sample. Scale up and subsequent XRPD analysis showed that the tolnaftate precipitate, after a given stir time and growth to larger sizes, was crystalline and matched that of the starting material. None of the methods investigated were found to be suitable for determining the crystalline form of the nanoparticles themselves. The work is, therefore, deemed incomplete and as such is not written with a view for publication.

7.2 Experimental

7.2.1 Materials

Tolnaftate (T-6638) was purchased from Sigma-Aldrich. Compound purity confirmed by NMR (>99%).

The DMSO used was UV-Vis grade, purchased from Fluka.

KH_2PO_4 and $Na_2HPO_4.2H_2O$ were purchased from Fisher Scientific.

7.2.2 DMSO stock, buffer and drug suspension preparations

Weighed stock solutions of nominal concentrations of 1 mM and 5 mM were prepared in DMSO for each individual compound (actual stock solution concentrations ranged from 0.99 to 1.03 mM, and 4.97 to 5.08 mM). These solutions will be referred to by their nominal concentrations. For Raman analysis, stocks in acetone were prepared in a similar manner.

A pH 7.0 (± 0.05) phosphate buffer was prepared, using 0.0025 moles of $KHPO_4$ and 0.0025 moles of Na_2HPO_4 per litre of deionised, Milli-Q water. The pH adjustment was conducted with 2 M NaOH if required. For this analysis, the buffer was then filtered through a 0.22 µm hydrophilic PTFE (Millipore) or a 0.1 µm VVLP filter (Millipore).

For a 1% DMSO (by volume) 1 mL suspension of drug concentration 50 µM, 10 µL of the 5 mM tolnaftate DMSO was pipetted into a microcentrifuge tube. 990 µL of filtered pH buffer was then added. The next step in sample preparation was dependent on the analysis technique. For samples (1 mL volume) analysed using the Raman microscope, a strip of aluminium foil was placed inside the microcentrifuge tube prior to addition of the solutions. After addition of the buffer aliquot, the samples were centrifuged at 9,300 x g rcf for 5 minutes. The tubes were placed

inside the centrifuge such that the precipitate was centrifuged onto the piece of film. This was then analysed under the Raman microscope. For scale up samples for XRPD, the appropriate larger volumes of DMSO stock and buffer were used. The scale up samples were stirred on either a Fisher FB15001 or a Stuart CC 162 stir plate. After the appropriate stir time, the samples were filtered through a 0.1 µm VVLP filter, and the dried powder offered to analysis.

7.2.3 Instrumental Details

i. Raman

Raman Microscopy (solid and precipitated onto foil samples)

The analysis was performed using a Leica DM/LM microscope equipped with a Leica 50 x/N.A. 0.75 objective lens. A Renishaw inVia with a 514.5 nm Ar+ Modu laser was used as the excitation laser source. Spectra were acquired for 5 or 120 s depending on the sample.

Raman probe (liquid samples - tolnaftate in DMSO and acetone, solid samples – tolnaftate starting material)

Avalon Instruments Ramanstation R3, 532 nm excitation, with a fibreoptic probe.

ii. NTA and NanoSight

I mL aliquots of the scale up 500 mL samples were removed during stirring and, prior to final filtration of the sample, analysed at various time points by NTA. The camera settings used were 'autosettings'. All processing settings were set to auto apart from 'blur' which was increased to 5x5.

iii. XRPD

A small quantity (10-50 mg) of the sample was analyzed using transmission foil XRPD data collected on a Bruker AXS D8-Advance transmission diffractometer equipped with θ/θ geometry, primary monochromated radiation (Cu $K\alpha_1$, λ= 1.54056Å), a Braun 1D position sensitive detector (PSD) and an automated multi-position *x-y* sample stage. The sample was mounted on a 28 position sample plate

supported on a polyimide (Kapton, 7.5 μm thickness) film. Data was collected from the sample in the range 4-35^{o} 2θ with a 0.015^{o} 2θ step size and 1s step^{-1} count time. Count time was increased to 5s step^{-1} if very small (less than 10 mg) amounts of sample were analysed.

7.3 Results and Discussion

7.3.1 1 mL scale analysis

It became apparent whilst performing scoping experiments that the analysis was not going to be straightforward. Several factors contributed to this. The concentrations used here are very low – in a 1 mL sample, there is roughly micrograms of solid material precipitating. This is, for many techniques, way below the limit of detection. Since we were particularly interested in how the process occurs at these low concentrations, increasing sample concentration was not an option. Table I documents the various analytical techniques considered for determination of the crystallinity of the drug nanoparticles formed for a typical 1 mL scale sample.

Table I: Analytical techniques considered for form analysis

Analytical Technique	Pros	Cons	Attempted?	Outcome
XRD	Gives definitive answer on crystallinity and polymorphic form.	Requires mg of sample	Yes	Could not separate nanoparticles from solution in large enough quantities for analysis on a mL scale.
Environmental-SEM	Can analyse sample in mother liquor, no need for separation.	Only gives morphology.	No	Advised that sample conc. is too low, and water to drug ratio too high.
Atomic Force Microscopy	Works in nm size range, can get down to atomic level resolution.	Only gives surface topography.	Yes	No signal from tolnaftate sample.
Differential Scanning Calorimetry	Gives information on glass transitions/phase transformations.	Requires roughly mg of sample.	No	Advised by expert it would be unlikely that a signal would be obtained.
PLM	Can distinguish amorphous from crystalline.	Small amounts difficult to analyse, cannot see individual nanoparticles in conventional light microscope.	Yes	No nanoparticulate results obtained.
Raman	Can potentially distinguish crystalline and amorphous material due to shifts in spectra.	Requires fairly large difference in spectra between molecules in a solution environment and those in crystalline lattice.	Yes	Some data obtained, however not conclusive (further details given later).

Both the small scale and low concentrations used contribute to the difficulty in finding a suitable analytical technique, as for many of the instruments, the amount of compound present in these samples is below the detection limit. An obvious solution to this problem would be to retain the concentration, but increase sample volume, to give an increase in drug – this will be discussed in detail in section 7.3.3 and presented its own difficulties.

7.3.2 1 mL scale analysis - Raman

An approach which had some (modest) success was the use of Raman spectroscopy. The application of Raman to investigate polymorphic forms of various drug molecules is well documented in literature.[5, 6, 7] The idea behind the use of Raman here was to firstly obtain reference samples of tolnaftate completely dissolved in solvent (adjusting for solvent scattering) and samples of the solid, crystalline starting material. It would then be possible to determine whether the precipitated material spectra matched that obtained from the starting material (crystalline) or was closer to that obtained from the dissolved drug reference spectra (representative of an amorphous sample). 1 mL scale samples of tolnaftate were used, and the initial precipitate was analysed. The nanoparticles were centrifuged onto a piece of metal foil, which concentrated the particles onto the surface and allowed a signal to be obtained using Raman microscopy. Removal of the mother liquor after centrifugation ensured the particles were kept as close to their original precipitating form as possible (i.e. lowered the risk of solution mediated phase transformation). The following spectra were obtained from the nanoparticulate material and the solid starting material reference;

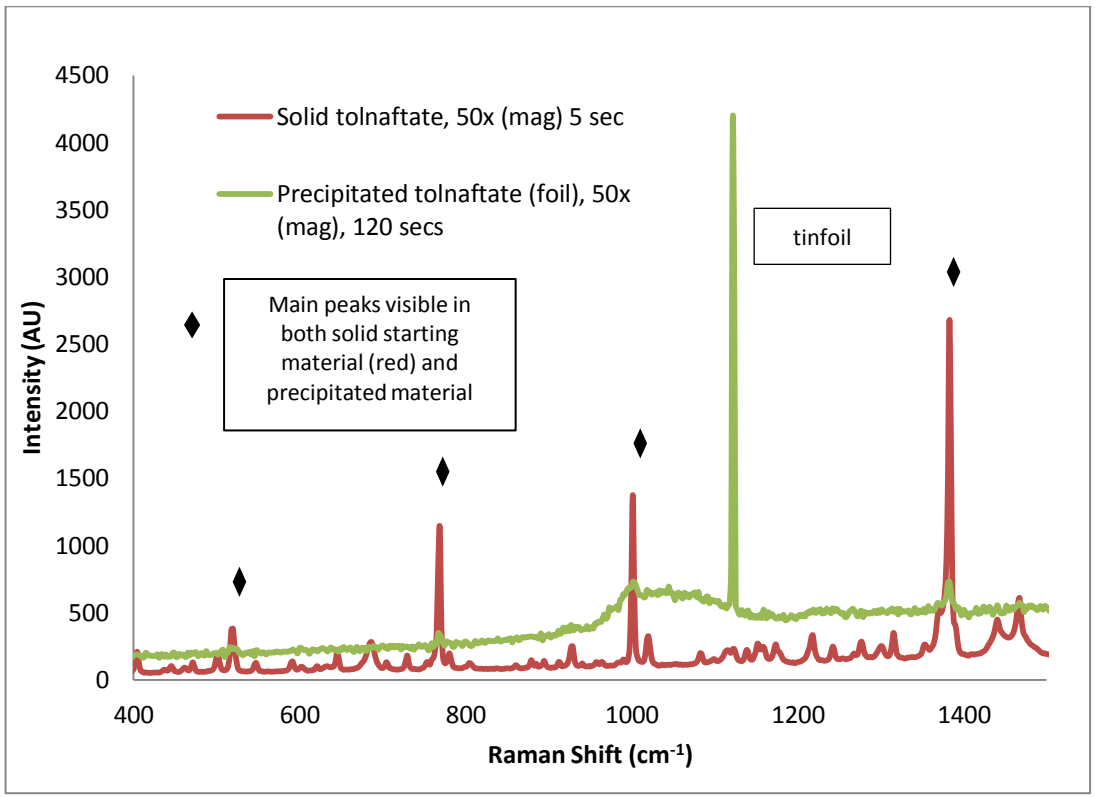

Figure 1: Overlay of precipitate and starting material Raman spectra. Large green peak is tinfoil peak.

From figure 1, the position of the four major peaks (indicated on the spectrum) from the crystalline starting material (red) match the weak peaks seen from the precipitated material (green). Note the very intense green peak is scattering from the foil. This demonstrates that Raman has the sensitivity to analyse the small, microgram amounts of material produced by precipitation on a 1 mL scale. In order to obtain crystallinity information from the results obtained here, at least one of the four main peaks indicated on figure 1 must show a shift when comparing solution and solid state Raman spectrum. Shown in figure 2 is an overlay of solution and solid state Raman spectra for this compound.

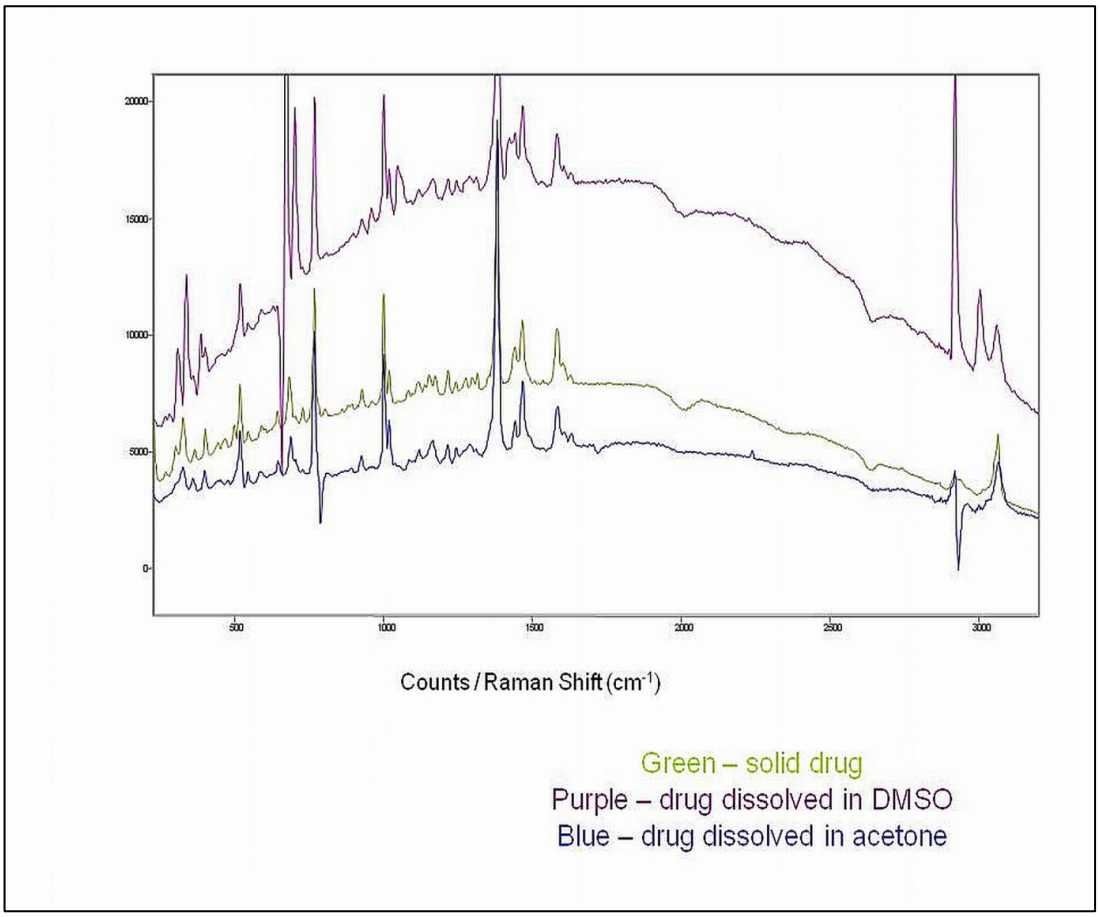

Figure 2: Overlay of Raman spectra of solution phase and solid drug

From figure 2, we can see clearly that the solution and solid Raman spectra of the drug were actually very similar. The four major peaks, one of which we needed to shift in order to obtain crystallinity information, have not moved. In fact, only one minor peak shifts very slightly between the two phases (see figure 3 for details). The solution spectra were obtained in both DMSO and acetone, with the solvent background spectra being subtracted from the respective sample spectra. Figure 3 shows a close up of the one minor peak which shifts slightly between the solid and solution phase. Note this is only visible when comparing the drug dissolved in acetone and the solid form (with drug samples dissolved in DMSO, an interfering peak from the DMSO masks the drug peak shift).

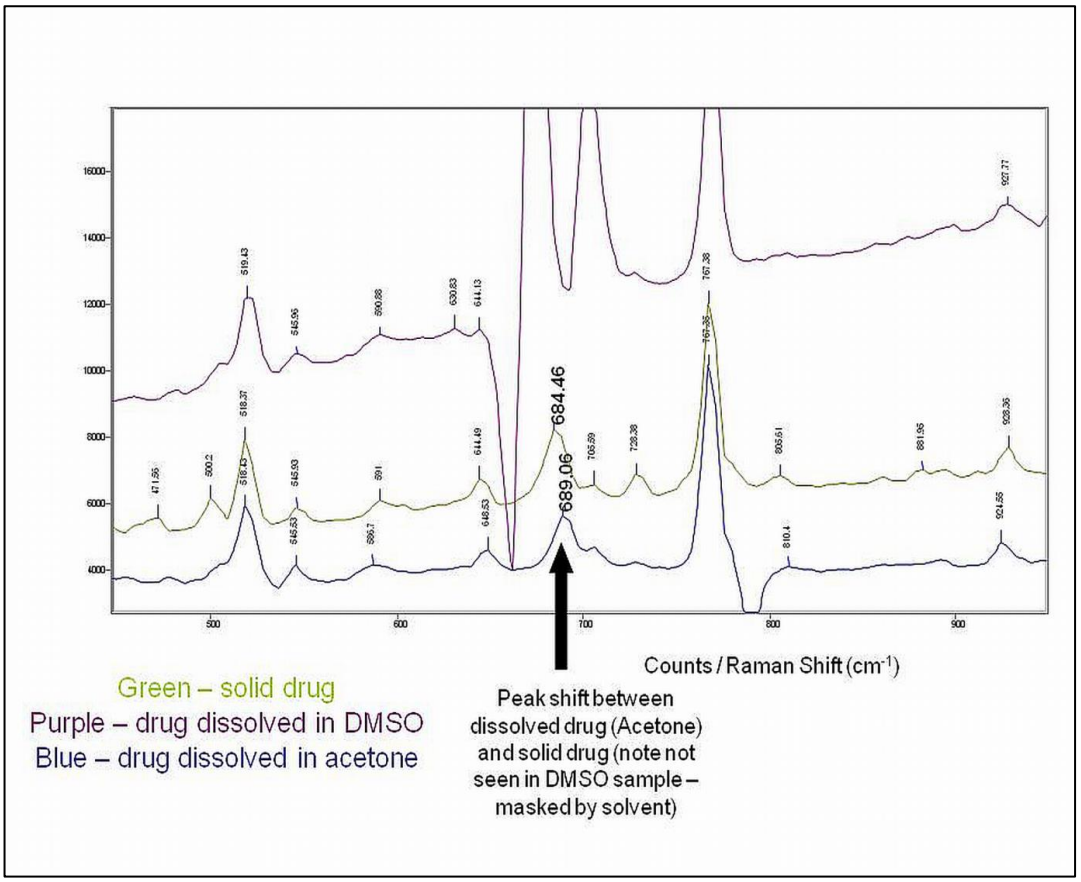

Figure 3: Overlay of Raman spectra showing shift between solution (acetone) and solid tolnaftate samples

Even with copious amount of sample, differentiation between the two states of matter would be difficult due to the similarity of the spectra of two phases. Therefore, using the small amounts typical of the experiments here makes it near impossible to obtain the required crystallinity information for this compound, and as expected, this minor peak was not visible in the precipitated sample spectrum (figure 1). An attempt was made to monitor the precipitate as it forms in suspension using the Raman probe, but no signal from the drug could be obtained. Whilst the results obtained do not address the question of the crystallinity of the nanoparticles, Raman itself does have the capability to analyse these small amounts of material (roughly micrograms for a 1 mL scale). Different compounds may be more successful, depending on the Raman shifts present and how different the solution and solid spectra were.

7.3.3 Scale up (500 mL) and XRPD analysis

As demonstrated, use of 1 mL volumes was problematic when attempting to perform physical form analysis of the precipitate, as only micrograms of material are present within a 1 mL volume. Larger scale samples at the same concentration offer the advantage of increased amounts of precipitate for analysis, potentially facilitating analysis. There is, however, the potential for the scale of the suspension prepared to affect the result obtained, due to varying surface to volume ratios. Jadhav et al noted that changing from a HTS analysis (1536 well plates) to a 96 well plate format affected the ability of certain compounds to form aggregates. They partly attributed this to the high surface to volume ratio for the 1536 well plate analysis. [8] The potential difference between differing sample scales was therefore kept in mind when performing the experiments and interpreting the results.

The samples were scaled up to a 500 mL sample volume, with a 50 µM concentration, as shown in Figure 4.

Figure 4: Tolnaftate 500 mL suspension (scaled up sample)

The scaled up samples were immediately (visually) turbid upon addition of the buffer to the DMSO stock solution. As with the lower volume samples, the scale up

samples contain nanoparticles suspended in the mixture, which grew over time. NTA analysis was performed on the scale up samples - we can compare the results obtained here to those detailed in chapter 4, to see the effects of scale on particle properties.

Table II: Comparison of tolnaftate 50 µM samples at 1 and 500 mL volumes, NTA data

Sample Scale (mL)	Mean particle diameter (nm) time 0	Mean particle diameter (nm) time 60	Mean particle conc. (10^6 particles/mL) time 0	Mean particle conc. (10^6 particles/mL) time 60	Mean calculated conc. of tol in nanoparticulate form, (µM) time 0	Mean calculated conc. of tol in nanoparticulate form, (µM) time 60
1	185 ± 19	635 ± 26	19.1 ± 3.0	2.8 ± 0.4	36 ± 16	>50
500	255 ± 17	326 ± 24	11.4 ± 2.0	8.0 ± 2.7	46 ± 6	>50

From table II, it can be seen that the 1 mL and 500 mL samples do give slightly different results from NTA analysis. Differences in both particle diameter and concentration are obtained; however, the amount of drug calculated to be present as nanoparticles is similar. The scale up samples do not seem to grow as much as the 1 mL samples, and have higher concentrations of smaller particles after the incubation period; however it should be borne in mind that the larger scale samples were stirred constantly whereas the 1 mL samples were not agitated during the incubation period. So this could be affecting the samples, rather than scale alone. It can be said that the calculated drug as particulate content is very similar for both sample volumes – the amount of drug which has precipitated does not appear to be different. The scale up samples, after a given stir time, eventually lead to large, individually visible particles. Due to the large volume used, this change could be monitored visually as well as using the NanoSight. Smaller volumes are not afforded this luxury. Whilst scale up meant that absolute compound quantities no longer presented any issue, it was not without its own difficulties. Ideally, once the particles were formed, they would be removed from the mother liquor immediately, dried, and then analysed by XRPD or any of the other techniques.

However, no filter membrane was found which would both remove the nanometre sized material from solution and allow recovery and removal of the precipitate from the filter paper. Since the particles were in the nm range, centrifugation of larger samples aliquots did not completely separate a sufficient quantity for analysis by any of the techniques. Thus whilst there was plenty of nanoparticulate material in the vial, the ratio of nanoparticle to liquid was such that the particles could not be isolated for analysis. However, over time, as the particles grew and ripened, there was a change from nanometre sized particles to micron sized particles. After the samples had reached this point, it was possible to filter off the solid material, and recover it for XRD analysis. This meant that whilst the initial aim of the scale up (crystallinity analysis of the nanosized precipitate) could not fulfilled using this method, analysis of the larger precipitate which developed over time was straightforward. The dried precipitate was removed from the filter paper of four samples and analysed using XRD. In all four cases, the samples exhibited diffraction, indicating crystallinity, and the powder pattern obtained matched that of the starting material. An example of the diffraction pattern overlaid with the starting material is shown in Figure 5.

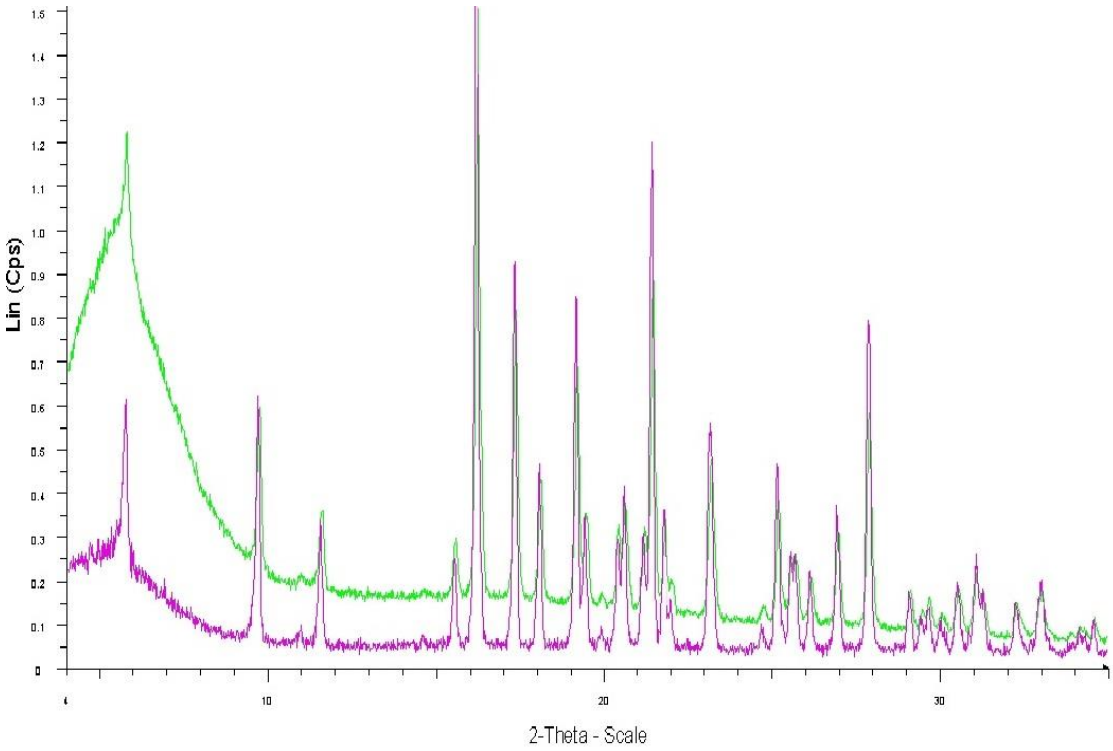

Figure 5: Powder XRD pattern from starting material (pink) and example scale up samples (green)

This means that, after several hours stirring, the precipitate is crystalline, and in the most stable form.

The kinetics of particle growth (from nanosized to larger, micron (visible) sized) showed large variation between samples, despite constant stirring. This could be observed as demonstrated by Figures 6 and 7.

Figure 6: Sample 1, 2.66 hours stirring

Figure 7: Sample 2, 2.66 hours stirring

The samples were prepared simultaneously, and the images captured after nearly 3 hours stirring, however sample 1 is visibly more cloudy/turbid than sample 2. It should be noted that while sample 2 looks 'clearer' than sample 1, it is still a suspension and has particles present. However, the particle sizes present in the two samples were different - sample 2 had larger, individually visible particles whereas sample 1's particles were still small (few hundred nm) at the point the image was captured. This gave the impression the sample looked 'clearer'. From 8 replicate samples, the following times for a visible improvement in sample 'clarity' were seen (table II);

Table II: Range of timescales for large particle formation

Sample	Time until 'clear' (hours)
A	2.5
B	3.3
C	5
D	5
E	7
F	4.5
G	3.3

Note D and E were prepared simultaneously.

As can be seen from table II, there is substantial variation in the amount of time taken for larger, visible particles to form, ranging from 2.5 to 7 hours. This large variability in the time taken for the nanoparticles to transform into larger particles is unusual; whilst nucleation is a random and chance event, this has already occurred as the nanoparticles have already formed, and in large numbers. Further growth or ripening of the particles would be expected to occur fairly consistently between samples – this is not the case. This suggests that there may be a second energy barrier to overcome, i.e. a second nucleation event/phase transition is required to allow growth into the larger, crystalline particles – and since nucleation is a stochastic process, this would go some way in explaining the variation in the data. From a phase transition point of view, it is possible the samples could contain varying low levels of larger, crystalline particles, which nucleate after the appearance of the bulk nanoparticle precipitate. The number of these rare, larger particles could poetentially govern the kinetics of the phase change –those samples with higher numbers of these larger particles would have higher rates of transformation, with phase change occurring more slowly in those samples with comparatively smaller numbers of crystalline particles. Following Ostwald's Rules, if

a phase change were to occur, the initial precipitate would be of a higher energy, such as an amorphous material or a higher energy polymorph. The two-step theory of nucleation could also be applied here; this theory states that nucleation is a two step process, with the first stage being that of the formation of 'clusters', which are highly disordered and liquid like. These clusters then rearrange to form a crystalline nucleus, although the sizes are likely to be smaller than those of the nanoparticles seen in this work. [9]

7.4 Conclusions

The form analysis of the nanoparticulate material formed by tolnaftate was not straightforward. Several different analytical techniques were considered, and two (Raman and scale up followed by XRPD analysis) implemented.

Raman microscopy demonstrated the capability to obtain a signal from a 1 mL sample of nanoparticles. Information on the crystal form of the particles could not be obtained. This was due to the extremely similar reference spectra obtained from crystalline solid and dissolved tolnaftate solution samples. Only one minor peak showed any shift between solid and solution samples; this small peak could not be resolved from the baseline in the precipitated sample. This is of course an issue with the compound rather than solely the limits of the instrumentation; other compounds could potentially show more success. Whilst this approach did not fulfil the initial aims of the experiment, further work on Raman with different compounds could be worth investigating.

Scale up of the samples overcame the problem of the low absolute amount of precipitated compound. It did, however, come with its own issues. Whilst the samples were visually immediately cloudy and had high concentrations of nanoparticles present, these could not be isolated from the mother liquor for immediate analysis. Due to their small size, neither centrifugation nor filtration was effective. It was noticed that, after several hours stir time, the particles had grown to a size which allowed them to be filtered, dried and then analysed by XRPD. These larger particles were the same polymorph as the solid tolnaftate starting material. An interesting observation was that the time taken for the scale up sample particles to grow to visible micron size particles was not consistent between samples. Even those prepared simultaneously could look visibly different. A few possible causes were discussed, however the underlying mechanism is not fully understood.

Future work could include further investigation of Raman to fully explore its potential for analysis of other compounds, and investigation of scale up with other compounds to see if the same issues are obtained with them as with tolnaftate.

7.5 References

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8. Overall Conclusions and Future Work

The work presented in this thesis has laid foundations for further detailed work into compound precipitation from DMSO stock and buffer mixtures. Whilst only three compounds have been studied here, several common behaviours are seen. All three compounds readily form nanoparticles upon mixing the stock and aqueous portion, even at concentrations close to their equilibrium solubility (at low supersaturations). Complete separation of the drug nanoparticulate and dissolved drug phases for quantification is problematic due to the small size of the particles (chapter 4). The particles grow and ripen over time, with the rate at which this occurs depending on the compound, the % DMSO present in the samples and the concentration of dissolved protein present (chapters 5 and 6). For one compound, the exact way in which the two solvents are added together has no effect, but subsequent mixing does (chapter 3). The particles formed by this particular compound eventually grow to large, micron sized particles which have been confirmed to be fully crystalline. No crystallinity information was obtained for any compound on the initial nanoparticle precipitate (chapter 7).

The experiments were performed with industrial relevance in mind. The factors investigated here are all variables in screening bioassays/kinetic solubility assays – thus the results here have direct relevance to these areas of drug discovery. Recommendations based on this work however differ based on the specific area in question. While screening bioassays and kinetic solubility assays have similar procedures and both utilise DMSO stocks, their ultimate aims are slightly different.

In a bioassay setting, it is desirable for the test compound to remain solubilised for as long as possible in order to fully assess its activity against the target. Precipitation on the timescale of the assay can result in both false positives and negatives; thus avoidance of precipitation completely is ideal. In accordance with this, it would be sensible, based on the results presented here, to recommend using higher % DMSO and protein concentrations, which slow down particle formation kinetics. It should however be kept in mind that these measures only have a kinetic effect and do not

prevent particle formation; as such analyses could still be susceptible to false positives due to non-stoichiometric inhibition. However, compared to lower percentages of DMSO and samples with no protein, more compound remains dissolved, and this could be an advantage. As mentioned in chapter 6, a possible way of eliminating false positives due to aggregation could be alteration of the dilution procedure – though further work is needed to ascertain whether the result seen here is mirrored in screening assay results.

The aim of a kinetic solubility experiment is to obtain an early indication of a compounds aqueous solubility without performing laborious thermodynamic shake flask experiments. While it is primarily used as a rough guide to solubility, the closer the results obtained are to the compounds' thermodynamic values, the better. From this point of view, the more rapid the precipitation, the better. So for example using lower percentages of DMSO and ensuring no protein is present would allow this to happen more quickly. Due to the small size of the particles which can potentially be formed, extra care should be given to the separation process employed (if required). The results of kinetic solubility assays can also be used to aid interpretation of bioassay results – ideally, for this purpose the experimental conditions should be as similar as possible. This could prove difficult based on the recommendations here, due to the differing aims of the experiments.

In terms of future work, detailed studies of this type on more compounds would be beneficial in determining the prevalence of the effects in a larger samples set. Several other factors which were only considered in scoping experiments here could also be looked at in detail. These include the size of the precipitation container used, the effect of varying drug concentrations, and performing the precipitation in 96/384 -well-plates. In terms of understanding non-stoichiometric aggregate based inhibition, an investigation into the effects of different proteins and more compounds, investigated from a more physical chemistry point of view, would be useful. It would also be interesting to see if changing the dilution procedure

affected the results of an inhibition experiment in a similar manner to how it affects the precipitation experiment seen here.

Whilst there is ample literature on various kinetic solubility analysis and on HT bioassays, there is a need to combine the underlying principles of both to fully understand the precipitation. It would also be beneficial to find an analytical method suitable for determining the crystalline form of the nanoparticles – this would give further insight into the process as a whole.

9. Appendix

9.1 Chapter 1 Additional Data

Figures 1 and 2 - Amiodarone HCl starting material NMR and XRPD analysis

Figures 3 and 4 - Clotrimazole starting material NMR and XRPD analysis

Figures 5 and 6 - Tolnaftate starting material NMR and XRPD analysis

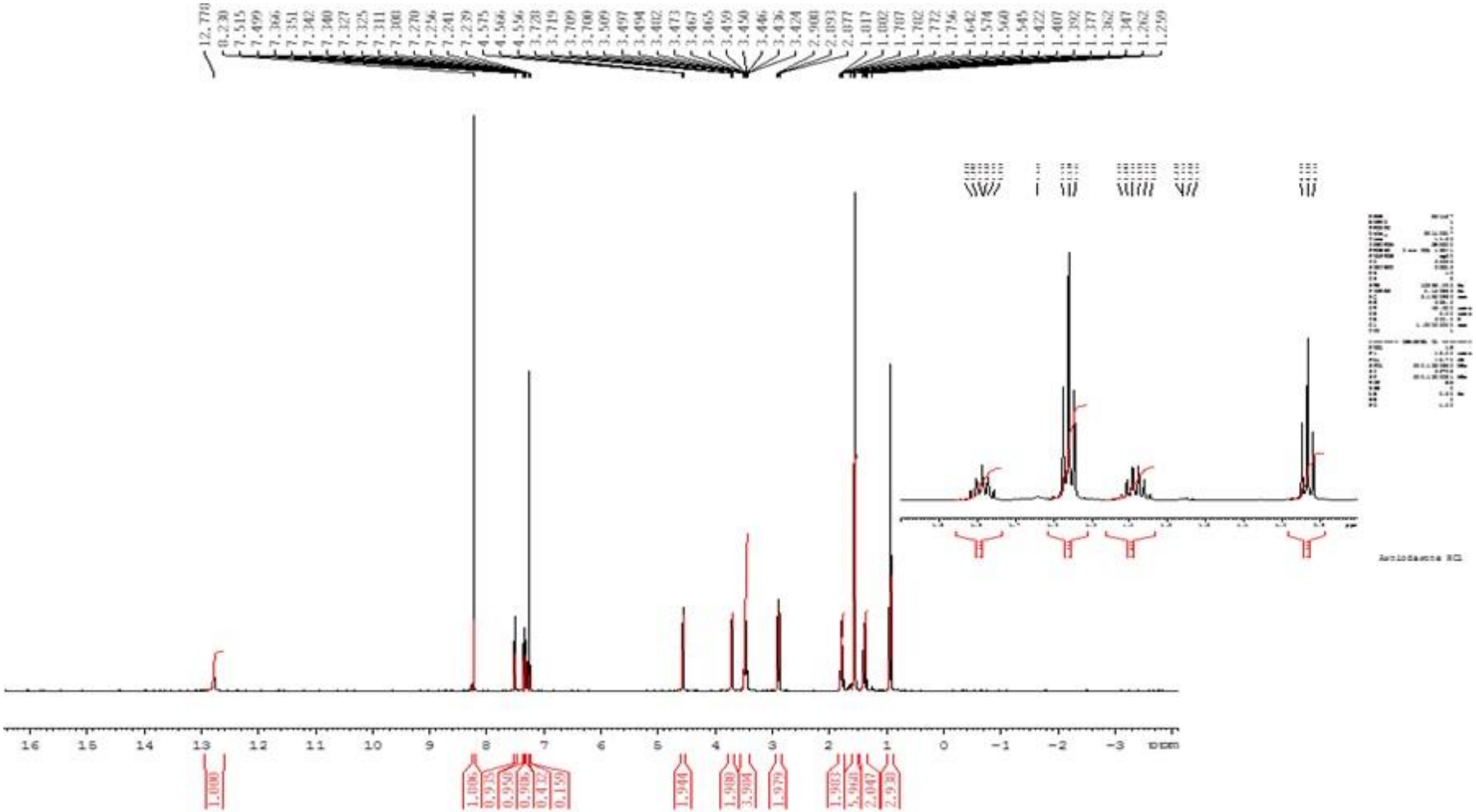

Figure 1: Amiodarone HCl, starting material NMR with close up insert of 0.9 to 1.9 ppm. Solvent used – $CDCl_3$

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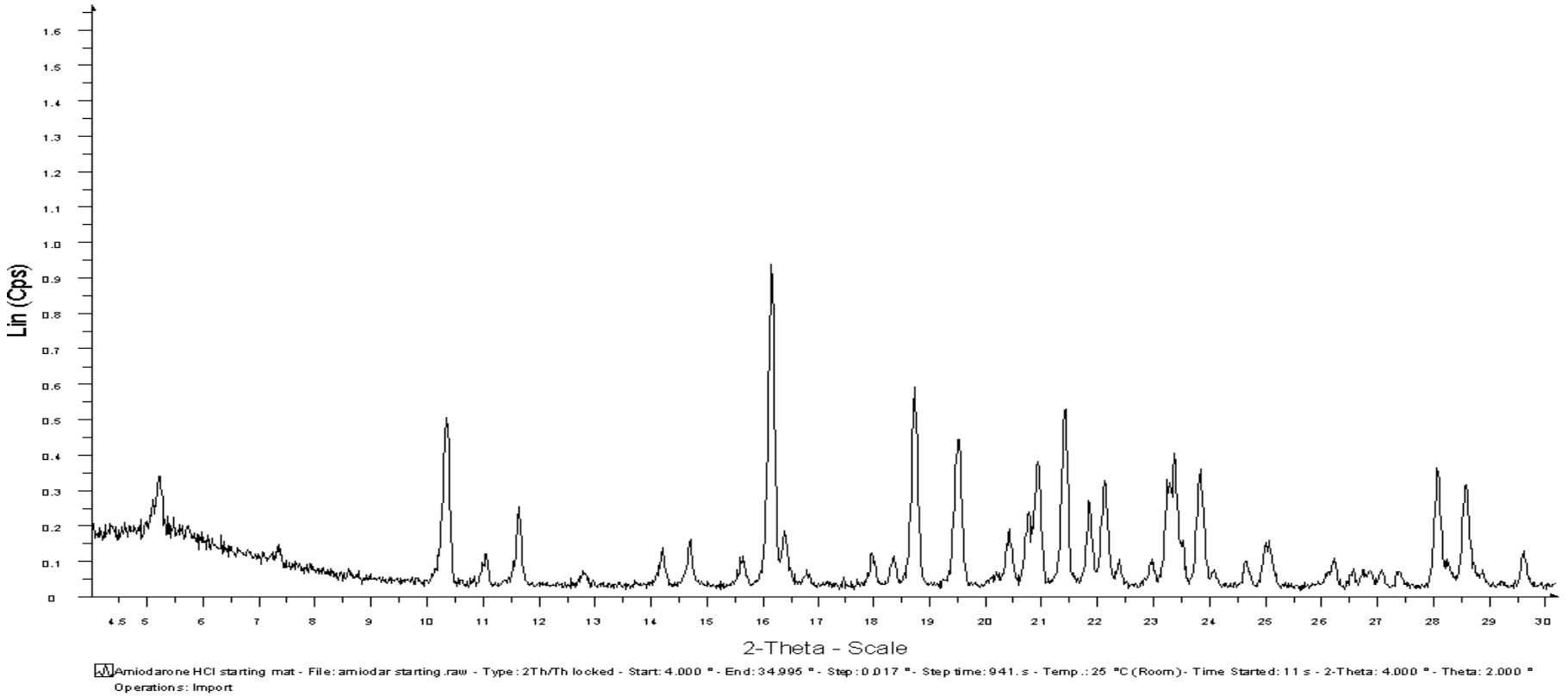

Figure 2: Amiodarone HCl XRPD trace of starting material

III

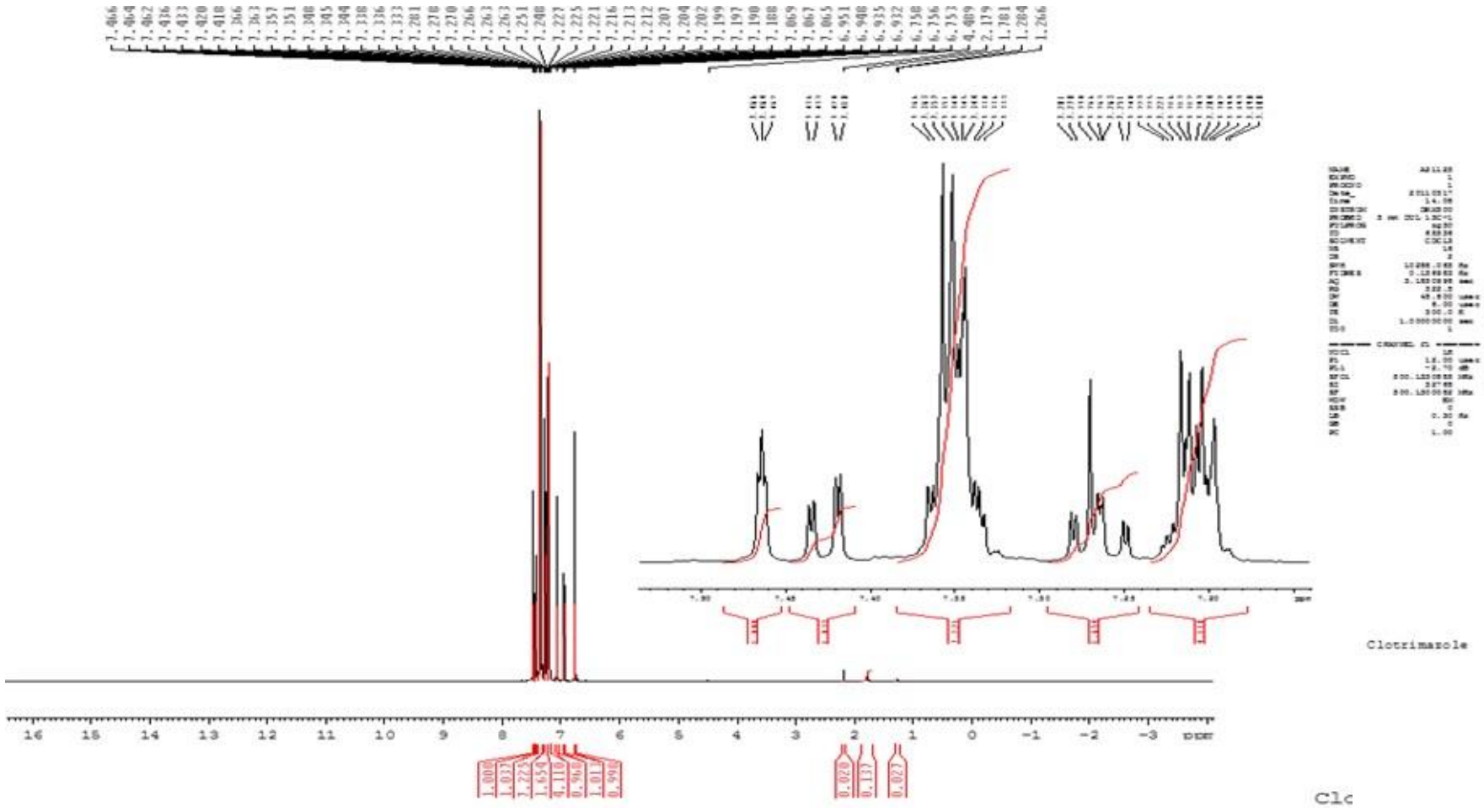

Figure 3: Clotrimazole starting material NMR, with close up insert of 7.00 to 7.90 ppm. Solvent used - $CDCl_3$

IV

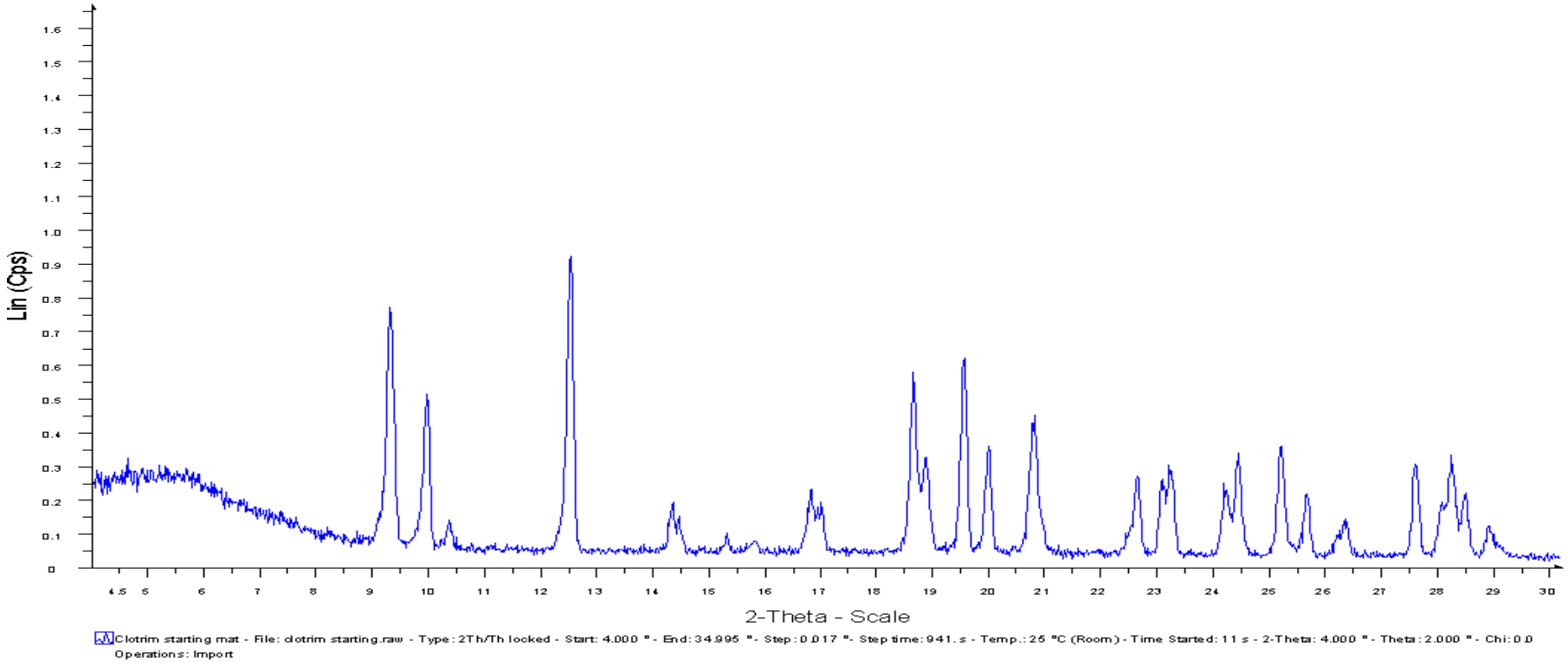

Figure 4:8 Clotrimazole starting material XRPD trace

V

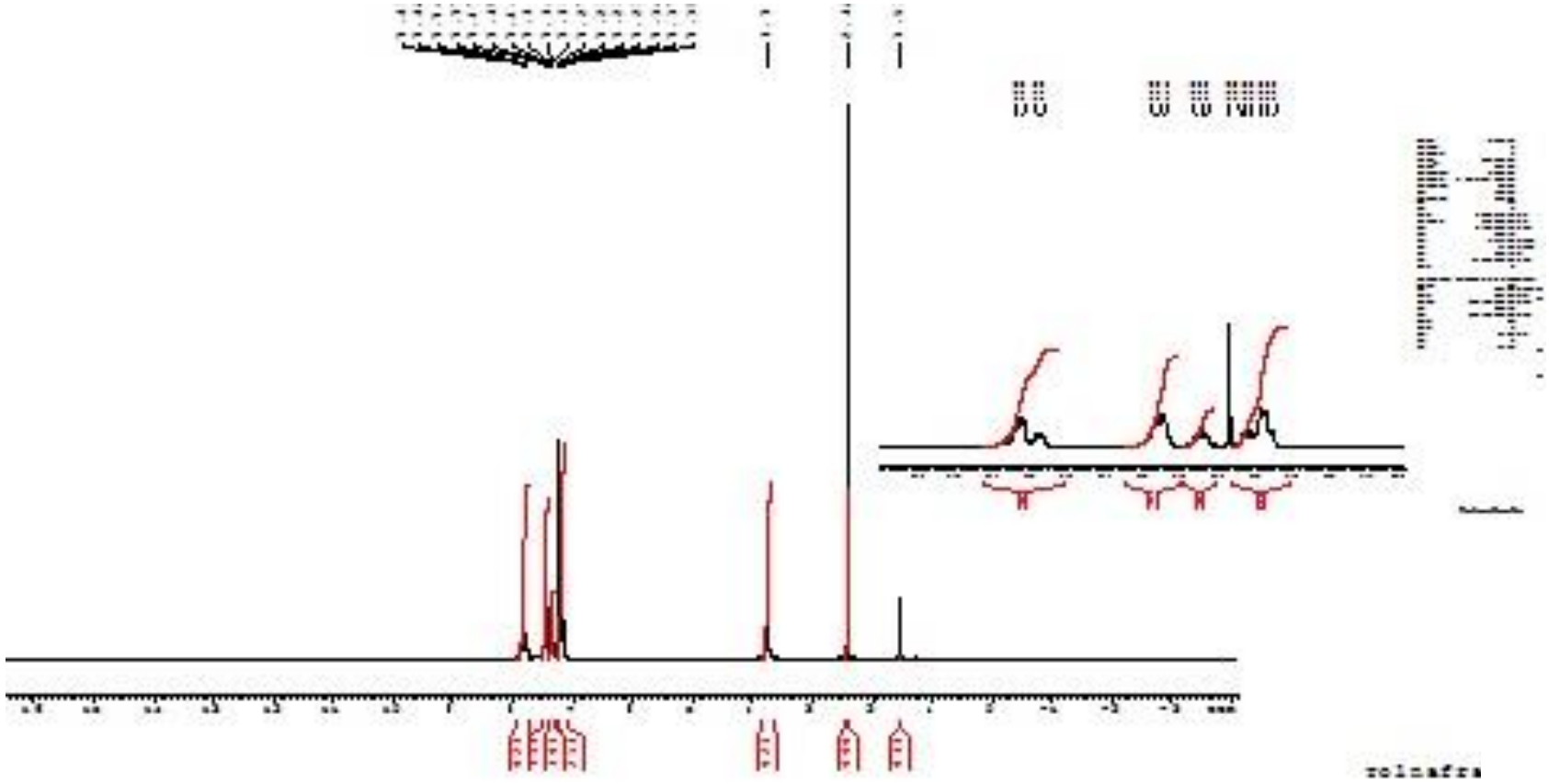

Figure 5: Tolnaftate starting material NMR, with close up insert of 6.90 to 8.00 ppm. Solvent used - $CdCl_3$

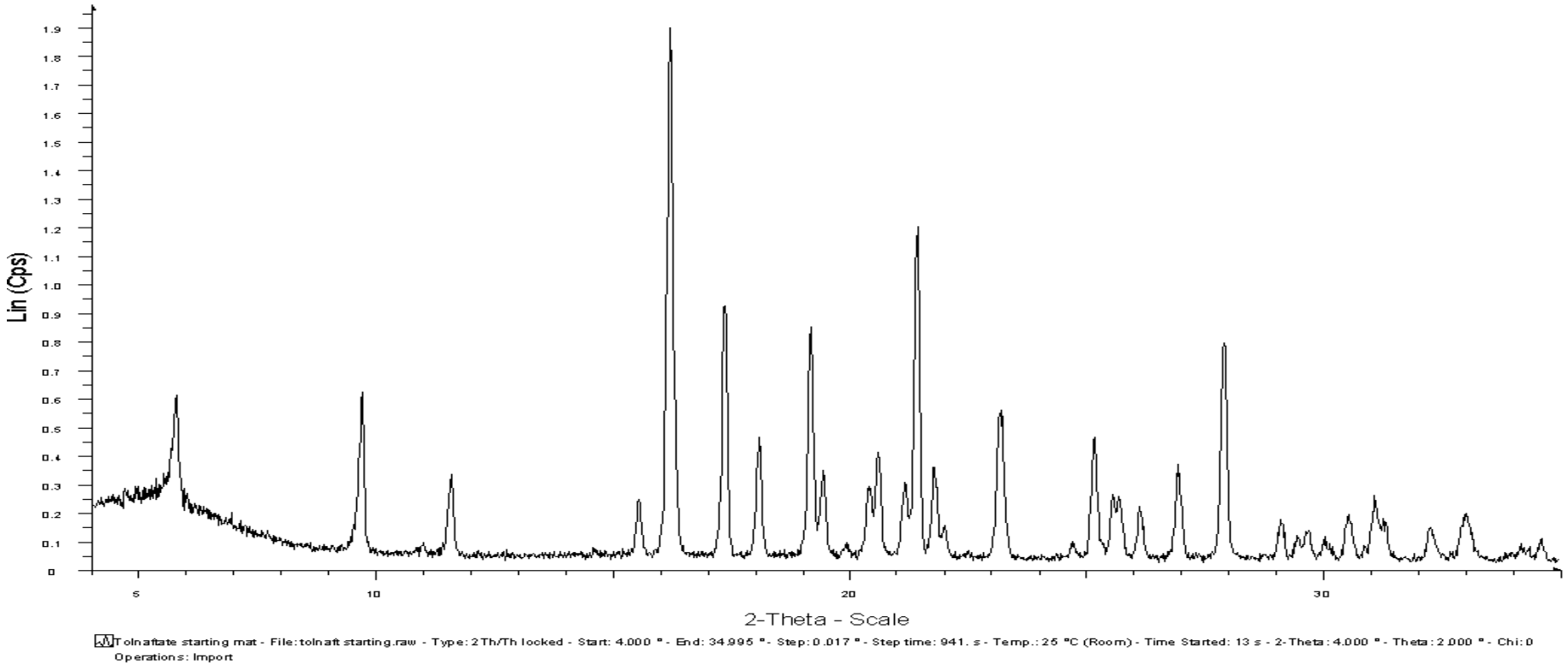

Figure 6: Tolnaftate starting material XRPD trace

9.2 Chapter 2 Additional Data

Table I and II – Amount of drug as particle calculation and Data, Rep 1

Table III and IV - Amount of drug as particle calculation and Data, Rep 2

Table V and VI - Amount of drug as particle calculation and Data, Rep 3

Table I: Amount of Drug in Particle Calculation, NanoSight Rep 1

Time (mins)	Volume Mean (adjusted not smoothed)	Concentration (E^8 particles/mL)	Diameter (cm) ^3	Volume Fraction	Vol Fraction X ρ X 1000 (g/litre)	Concentration of drug in particles (moles/litre)	No of Nanomoles	% Drug Added
0	127	13.08	2.05E-15	1.40E-06	1.71E-03	5.58E-06	5.58	18.66
5	145	14.15	3.05E-15	2.26E-06	2.76E-03	8.98E-06	8.98	30.04
10	147	10.73	3.18E-15	1.78E-06	2.18E-03	7.10E-06	7.10	23.73
20	158	11.35	3.94E-15	2.34E-06	2.87E-03	9.32E-06	9.32	31.17
30	190	7.39	6.86E-15	2.65E-06	3.24E-03	1.06E-05	10.55	35.30
60	238	5.51	1.35E-14	3.89E-06	4.75E-03	1.55E-05	15.47	51.73
90	260	5.09	1.76E-14	4.68E-06	5.73E-03	1.86E-05	18.63	62.30
120	282	3.66	2.24E-14	4.30E-06	5.25E-03	1.71E-05	17.09	57.16
190	322	2.16	3.34E-14	3.77E-06	4.62E-03	1.50E-05	15.01	50.22

Table II: Data From NanoSight Rep 1

Time(mins)	Number Mean Particle Size adjusted not smoothed (nm)	Standard Deviation	Concentration (E^8 particles/mL)	No of Completed Tracks	Error in Concentration (E^8 particles/mL)
0	108	46	13.08	756	0.48
5	126	50	14.15	630	0.56
10	124	56	10.73	579	0.45
20	140	53	11.35	733	0.42
30	170	60	7.39	602	0.30
60	213	76	5.51	447	0.26
90	229	86	5.09	374	0.26
120	244	96	3.66	288	0.22
190	299	85	2.16	136	0.19

X

Table III: Amount of Drug in Particle Calculation, NanoSight Rep 2

Time (Mins)	Volume Mean (Adjusted not smoothed)/ nm	Concentration/10^8 particles per mL	Diameter (cm) ^3	Volume Fraction	Vol Fraction X ρ X 1000 (g/litre)	Concentration of drug in particles (moles/litre)	% Of Drug Added	no of nanomoles
0	218	4.41	1.04E-14	2.39E-06	2.92E-03	9.51E-06	31.82	9.51
10	253	1.92	1.62E-14	1.63E-06	1.99E-03	6.47E-06	21.65	6.47
20	264	1.57	1.84E-14	1.51E-06	1.85E-03	6.01E-06	20.12	6.01
30	307	1.99	2.89E-14	3.01E-06	3.69E-03	1.20E-05	40.10	11.99
60	308	1.72	2.92E-14	2.63E-06	3.22E-03	1.05E-05	35.00	10.46
90	314	1.33	3.10E-14	2.15E-06	2.64E-03	8.57E-06	28.67	8.57
120	353	0.76	4.40E-14	1.75E-06	2.14E-03	6.96E-06	23.28	6.96
150	415	1.27	7.15E-14	4.75E-06	5.81E-03	1.89E-05	63.21	18.90
180	428	1.52	7.84E-14	6.24E-06	7.63E-03	2.48E-05	82.99	24.81

Table IV: Data From NanoSight Rep 2

Time (Mins)	Number Mean Adjusted Data not smoothed nm	Std Dev	Concentration (E8 particles/mL)	No of Completed Tracks	Error in Concentration (E8 particles/mL)
0	206	49	4.41	1516	0.11
10	240	54	1.92	642	0.08
20	251	56	1.57	444	0.07
30	289	71	1.99	505	0.09
60	291	69	1.72	425	0.08
90	299	66	1.33	300	0.08
120	326	90	0.76	166	0.06
150	373	125	1.27	257	0.08
180	378	138	1.52	293	0.09

Table V: Amount of Drug in Particle Calculation, NanoSight Rep 3

Time (Mins)	Volume Mean (raw)	Concentration (E8 particles/mL)	Diameter (cm) ^3	Volume Fraction	Vol Fraction X ρ X 1000 (g/litre)	Concentration of drug in particles (moles/litre)	% Of Drug Added	no of nanomoles
0	155	14.5	3.724E-15	2.826E-06	3.456E-03	1.12425E-05	37.48	11.24
10	243	1.92	1.435E-14	1.442E-06	1.763E-03	5.73616E-06	19.12	5.74
20	238	1.57	1.348E-14	1.108E-06	1.355E-03	4.40688E-06	14.69	4.41
30	281	1.99	2.219E-14	2.311E-06	2.826E-03	9.19333E-06	30.64	9.19
60	309	1.72	2.950E-14	2.656E-06	3.248E-03	1.05659E-05	35.22	10.57
90	315	1.33	3.126E-14	2.176E-06	2.661E-03	8.65534E-06	28.85	8.66
120	395	0.76	6.163E-14	2.451E-06	2.998E-03	9.75227E-06	32.51	9.75
150	389	1.27	5.886E-14	3.912E-06	4.785E-03	1.55652E-05	51.88	15.57
180	421	1.52	7.462E-14	5.936E-06	7.259E-03	2.36152E-05	78.72	23.62

Table VI: Data From NanoSight Rep 3

Time (mins)	Number Mean Adjusted Data not smoothed (nm)	Std Dev	Concentration (E8 particles/mL)	No. of Completed Tracks	Error in Concentration (E8 particles/mL)
0	134	55	14.5	2598	0.28
10	225	54	6.05	1357	0.16
20	215	74	5.12	878	0.17
30	260	76	2.55	620	0.10
60	282	90	2.23	549	0.10
90	284	97	2.07	400	0.10
120	355	120	1.62	326	0.09
150	373	125	1.27	257	0.08
180	383	138	1.52	293	0.09

9.3 Chapter 3 Additional Data

With both instruments, optimisation of pipetting settings was required for accurate dispension of the 10 µL DMSO aliquot. Slightly different optimisation was used for each instrument, as detailed below. Once optimised, reproducible 10 µL dispension was achieved.

1. Detailed robot settings

Lab 1 – Tecan Genesis

10 µL DSMO stock solution dispension -The setting used was the default 'DMSO low volume' setting (see appendix for details), with one change. The robot was set to aspirate 30 µL of the solution, discard 10 µL to waste, and then pipette 10 µL into the sample vial. This gave more reproducible results than pipetting 10 µL from a dry tip.

Aspiration settings;

Speed – 20 µL/s

Delay – 200 ms

Liquid detection used.

Aspirate position – z max ± offset, no tracking.

Dispension settings;

Speed – 600 µL/s

Break off speed – 400 µL/s

Delay – 0ms

Dispense position – z dispense ± offset, no tracking.

990 µL of buffer - default ‘water’ setting. For samples mixed by the robot, the instrument was

programmed to aspirate and dispense 500 µL of suspension 5 times.

Aspiration settings;

Speed – 150 µL/s

Delay – 300 ms

Aspirate position – z max ± offset, with tracking, 2mm X centre Y centre. Use liquid detection.

Dispension settings

Speed – 600 µL

Break off speed – 400 µL/s

Delay – 0ms

No liquid detection.

Dispension position- z dispense ± offset, no tracking.

990 µL of DMSO - the default ‘DMSO’ setting.

Aspiration settings;

Speed – 150 µL/s

Delay – 400 ms

Aspiration position – z max ± offset, no tracking.

Dispension settings;

Speed – 600 µL/s

Break off speed – 400 µL/s

Delay – 0 ms

Dispension position – z dispense ± offset, no tracking.

Lab 2 – Biomek 2000

10 µL DSMO stock solution dispension – The robot was programmed to aspirate at height 25%, using liquid level tracking, with an aspiration rate of 10. Pre-wet and tip touch settings were applied. The dispension settings were to dispense at 25% height, and dispension type was set to 'to deliver'. The rate used was 10, and the tip touch setting was applied.

990 µL of buffer – The robot was programmed to aspirate at height 50%, using liquid level tracking, with an aspiration rate of 10. Pre-wet settings were applied. The dispension settings were to dispense at 70% height, and dispension type was set to 'to contain', with a 'blowout'. It should be noted that Lab 2 experiments for 'Early' and 'Later' experiments (see section 3.2) were all performed with a dispension speed setting of 10 (estimated to be around 0.5 mL/second). This is the recommended setting for this instrument, however was not suitable for preparation of

large numbers of samples as it caused buffer to ‘splash’ out of the tube around 50% of the time. For this reason, the rate was lowered to setting 7 (estimated to be around 0.35 mL/second), and all other Lab 2 experiments (mixing investigation experiments, section 3.3 onwards) use rate setting 7990 µL of DMSO - The robot was programmed to aspirate using liquid ‘sensing’, with an aspiration rate of 10. Pre-wet settings were applied. The dispension settings were to dispense at 70% height, and dispension type was set to ‘to contain’, with a ‘blowout’. The rate used was 10.

2. Detailed HPLC Conditions

Lab 1

Chromeleon software (version 6.80 SR8 Build 2623(156243)) was used with the HPLC system.

A 2 mL/min flow rate was used, and the column temperature was 60 °C. An injection volume of 10 µL was used, and the detector wavelength set to 267 nm. The total run time was 3.5 mins. Mobile phase A consisted of 5 % MeCN in water + 0.1 % TFA, and mobile phase B consisted of 5 % water in MeCN + 0.1 % TFA. The retention time of the drug using this method was 2.44 minutes.

Table III; Gradient Conditions, Lab 1

Time (mins)	%B
0.5	40
2.5	95
3	95
3.01	40
3.5	40

Lab 2

Atlas software (version 1.6.0) was used with the HPLC system.

A 2 mL/min flow rate was used, and the column temperature was 40 °C. An injection volume of 10 µL was used, and the detector wavelength set to 260 nm. The total run time was 3.5 mins. Mobile phase A consisted of water + 0.02 % TFA, and mobile phase B consisted of MeCN + 0.02 % TFA. The retention time of the drug using this method was 2.18 minutes.

Table IV; Gradient conditions Lab 2

Time (mins)	%B
0	40
0.5	40
1.5	95
2.5	95
2.51	40
3.5	40

Lab 3

Empower Pro, 2002 (build 1154) software was used with the HPLC system.

A 1 mL/min flow rate was used, and the column temperature was 40 °C. An injection volume of 3 µL was used, and the detector wavelength set to 257 nm. The total run time was 3 mins. Mobile phase A consisted of water + 0.1 % TFA, and mobile phase B consisted of MeCN + 0.1 % TFA. An isocratic method was used. The retention time of the drug using this method was 1.99 minutes.

3. Dissolution from solid and kinetic solubility – detailed results

Table V: Dissolution from solid results

Time (hrs)	*0.66*	*24*	*48*	*162.5*
Amount of drug present in supernatant (nmoles/mL)	2/5 samples - no analyte peak detected. Mean from remaining 3 samples -0.8 ± 0.6]	4/5 samples - no analyte peak detected. Remaining 1 sample - 2.3	4/5 samples - no analyte peak detected. Remaining 1 sample - 0.3	5/5 samples - no analyte peak detected.

Table VI: Kinetics of precipitation results

Time (hrs)	*0*	*48*	*120*	*168*
Amount of drug present in supernatant (nmoles/mL)	2.7 ± 1.9	2/5 samples - no analyte peak detected.1/5 samples - poor recovery. Mean from other two samples - 1.7	3/5 samples - no analyte peak detected. Mean from 2/5 samples - 0.7	3/5 samples = no analyte peak detected. Mean from remaining two samples - 0.6

4. Lab 3 25 °C data

As with the other data sets, VOR mixing is significantly different to ASP-M and NO mixing, and returns the lowest supernatant amount.

Table VII: Lab 3 25 °C supernatant amounts and p-values

Treatment	Amount in supernatant, nanomoles ± S.D.		p value
50 nanomoles per mL	VOR, 9.9 ±3.0	NO, 13.8 ± 2.4	0.01
50 nanomoles per mL	VOR, 9.9 ±3.0	ASP-M, 15.1 ± 2.9	0.003
50 nanomoles per mL	NO, 13.8 ± 2.4	ASP-M, 15.1 ± 2.9	0.3 (NS)

9.4 Chapter 5 Additional Data

Table XIV -1 and 5% DMSO, supernatant/filtrate amounts after various separation procedures

Table XV- 1 and 5% DMSO samples, incubation times (supernatant analysis after centrifugation)

Table XIV: 1 and 5% DMSO, supernatant/filtrate amounts after various separation procedures

	Amount present in supernatant ± S.D., µM, after centrifugation (5 mins, 9,300 x g rcf)		**Amount present in filtrate ± S.D., µM, 0.45 µm PVDF filtration**		**Amount present in filtrate ± S.D., µM, 0.22 µm PVDF filtration**	
Compound	***1% v/v DMSO***	***5% v/v DMSO***	***1% v/v DMSO***	***5% v/v DMSO***	***1% v/v DMSO***	***5% v/v DMSO***
Amiodarone HCl	27.5 ± 1.53	40.0 ± 1.9	27.3 ± 2.7	36.9 ± 1.0	11.9 ± 0.8	25.3 ± 1.9
Clotrimazole	20.2 ± 3.2	31.2 ± 2.9	8.4 ± 0.6	19.5 ± 0.3	N/A	N/A
Tolnaftate	13.3 ± 5.6	31.2 ± 3.3	No Peak Detected	Peak Detected Inconsistently	N/A	N/A

Table XV: 1 and 5% DMSO samples, incubation times (supernatant analysis after centrifugation)

Incubation time, mins	**Amiodarone HCl, amount of compound present in supernatant ± S.D., µM**		**Clotrimazole, amount of compound present in supernatant ± S.D., µM**		**Tolnaftate, amount of compound present in supernatant ± S.D., µM**	
	1% DMSO	***5% DMSO***	***1% DMSO***	***5% DMSO***	***1% DMSO***	***5% DMSO***
0	27.5 ± 1.5	39.8 ± 2.1	20.2 ± 3.2	31.2 ± 2.9	13.3 ± 5.6	31.2 ± 3.3
10	23.8 ± 0.3	41.0 ± 0.5	-	-	***9.4 ± 6.7***	20.8 ± 3.3
60	22.9 ± 0.5	38.6 ± 0.5	16.5 ± 0.5	29.4 ± 0.5	-	-
180	-	-	18 ± 1	29 ± 2	***3.0 ± 0.5***	16.8 ± 0.2
1440	24.0 ± 0.3	37.0 ± 0.4	***2.9 ± 0.2***	*24.7 ± 0.6	****5.1 ± 1.8***	*17.4 ± 4.0

*poor recovery (<85 %)

9.5 Chapter 6 Additional Data

Figure 9 - Average particle size distributions for samples containing various protein amounts, time zero

Figure 10 - Amiodarone HCl, pH 4 samples, time zero, with and without protein present

Figure 11 - Amiodarone HCl pH 4 samples, time 60 mins, with and without protein present

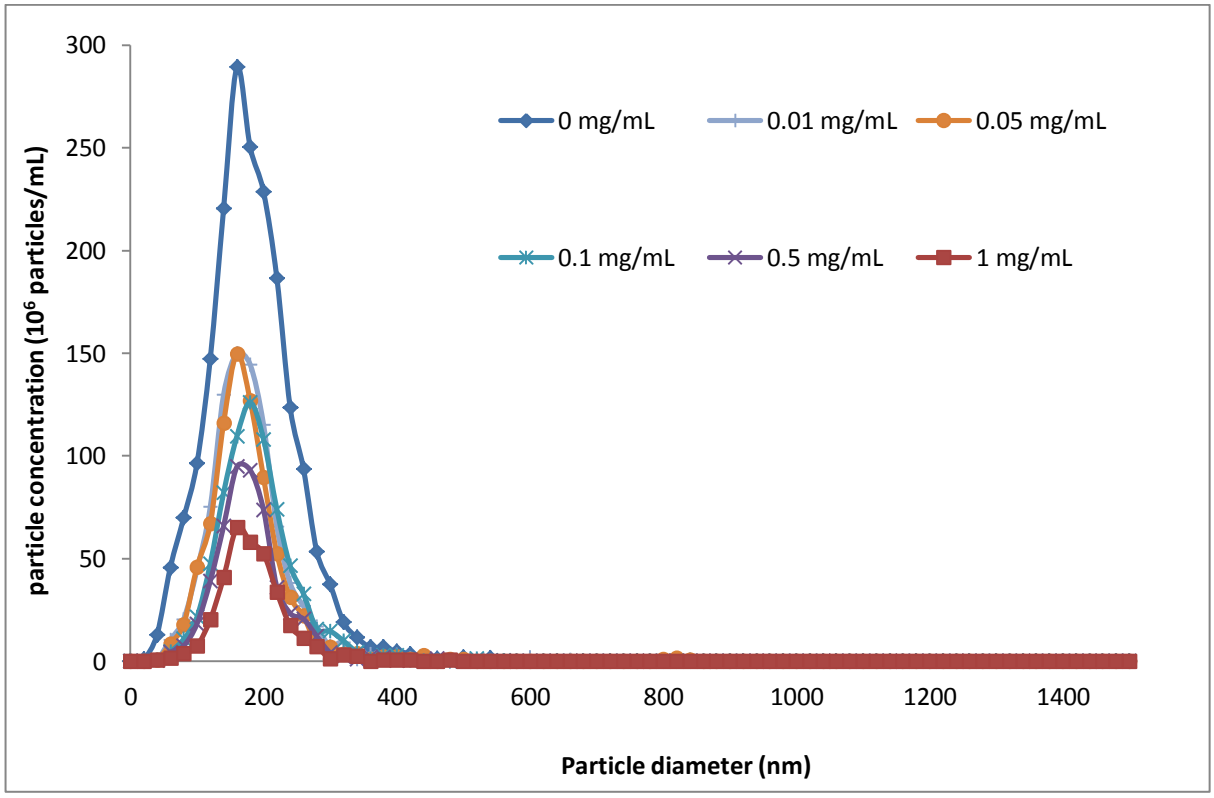

Figure14: Average particle size distributions for samples containing various protein amounts, time zero

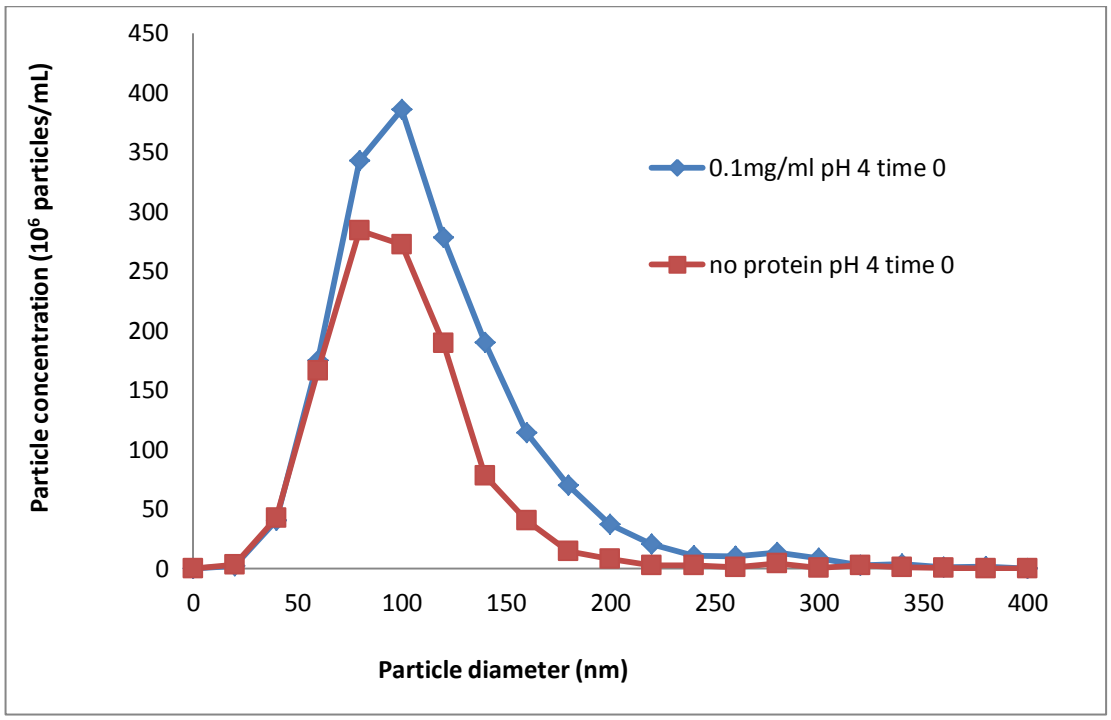

Figure15: Amiodarone HCl, pH 4 samples, time zero, with and without protein present

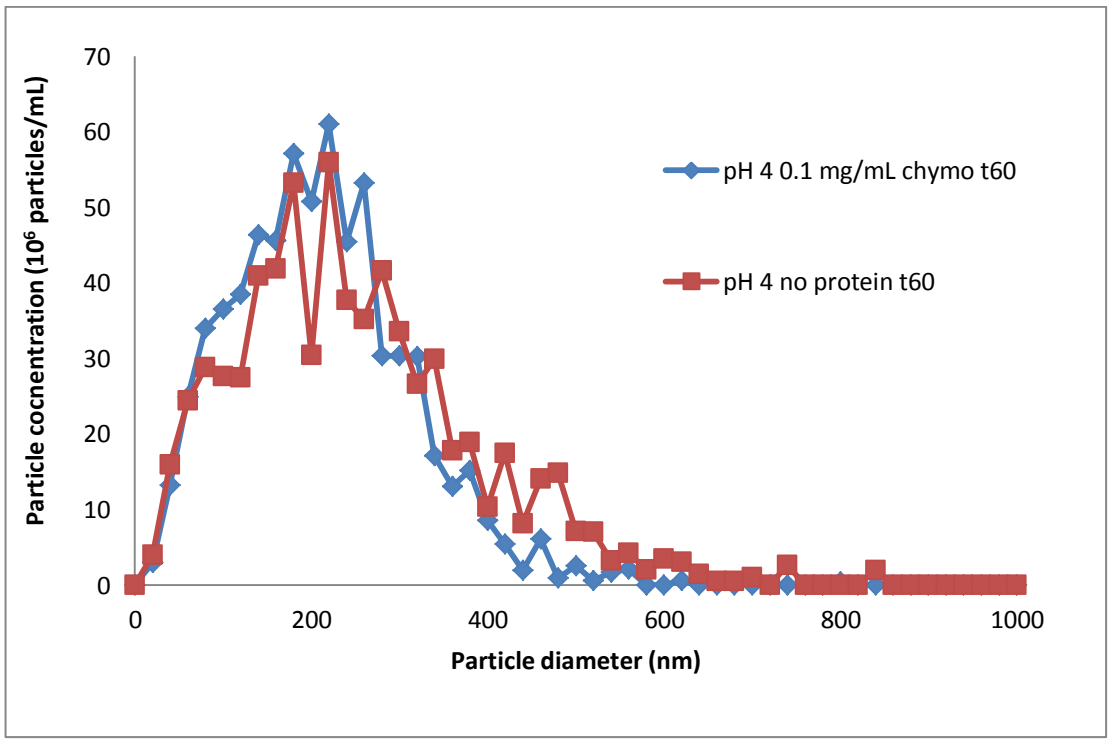

Figure 16: Amiodarone HCl pH 4 samples, time 60 mins, with and without protein present