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Solid-state epimerisation and disproportionation of pilocarpine HCl: Why we need a 5-stage approach to validate melting point measurements for heat-sensitive drugs.

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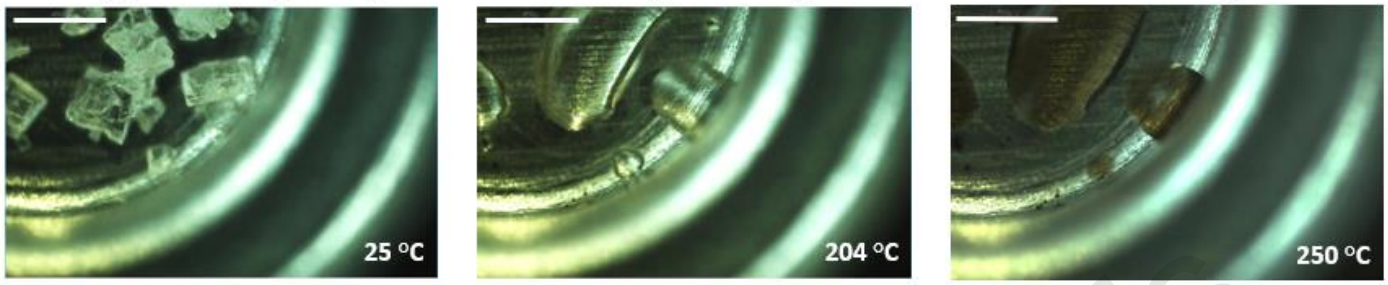
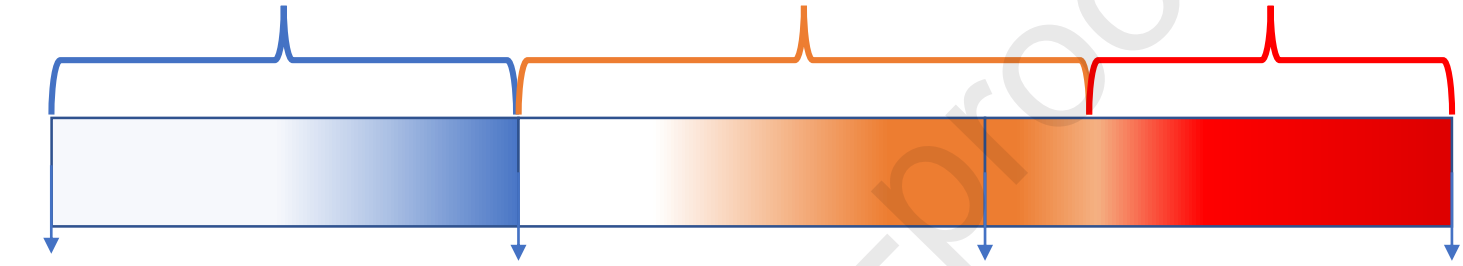
Keywords: Differential scanning calorimetry, melting point, heat-sensitive drugs, pilocarpine HCl, epimerization, disproportionation, thermal degradation.

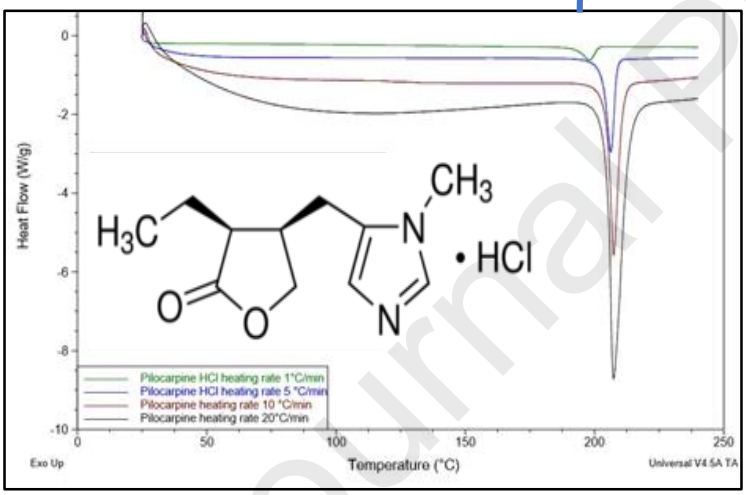
Melting process of pilocarpine HCl with heating rate 10 °C/min. Scale bars represents 700µm

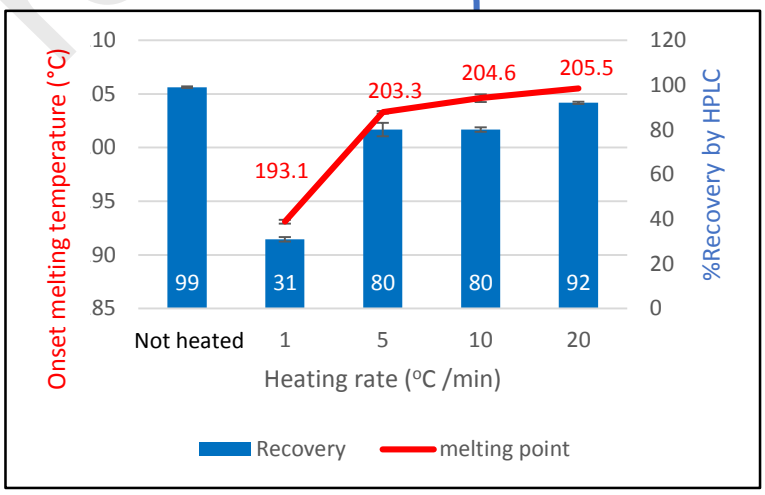


Isomerisation HCl evaporation Thermal degradation



25°C **100°C** **180°C** **250⁺°C**





1 Abstract

Melting points for new drugs are reported in regulatory documents, e.g. investigational brochures, and frequently in published research; however, the authors do not typically consider that heat-induced degradation can affect the melting point measurement. Applying a single heating rate is not adequate, and thus many melting points in the literature and regulatory documentation are not valid. Our aim was to validate a five-stage approach for the melting point measurement of heat-sensitive drugs. These stages are; 1) observe melting; 2) record mass loss; 3) measure melting points at different heating rates; 4) characterise degradation and 5) test for potential isomerisation. Applying this approach to pilocarpine HCl illustrated the sensitivity of a melting point to thermal degradation. Due to salt disproportionation & loss of HCl gas, pilocarpine's melting point decreased by 14^{o}C when the heating rate was lowered from 20 to 1^{o}C/min. Epimerization occurred before melting was reached. Increasing the heating rate diminished disproportionation; however, this did not remove epimerization. Thus, the melting point of pilocarpine HCl of 205.5$\pm$0.4^{o}C measured at 20^{o}C/min represents the melt of a racemic mixture containing inactive isopilocarpine. Heating above the melting point accelerated degradation, a rate of 5^{o}C/min recovered just 38$\pm$1% of pilocarpine. Such data predicted a shelf-life of 6.6years. Pilocarpine successfully validated the multistage approach by providing new knowledge concerning its thermal stability. Our 5-stage approach must be applied to all new drugs especially if their formulation requires heat. For example, thermal stability is an infrequently considered pre-requisite in the emerging field of 3D printing.

2 Introduction

Melting points (MPs) are critical material attributes used for the classification and the characterisation of crystalline drug substances; they reflect drug purity, drug quality, drug stability and inform medicine formulation strategies (Mathkar et al., 2009; Okorie et al., 2016). An investigational brochure, a prerequisite for the development of new medicine, requires the physical information that is relevant to the clinical development of the new product. Thus, an accurately measured melting point of the drug substance is typically included within a brochure.

The melting point of a crystalline drug is the temperature at which the solid is in equilibrium with its liquid. This definition assumes that at equilibrium, both the chemical composition and the polymorphic form of the drug do not change. When measuring melting points using a heating profile, the same assumptions apply; however, the chemical composition of a drug post melting is rarely recorded. In order to test these assumptions and potentially reduce the variance in the reported melting points for active pharmaceutical ingredients, the authors have developed a multistage approach. The aim of the work reported here was to validate this five-stage approach for the melting point measurement of a heat-sensitive drug. These five stages are; #1 visually observe melting; #2 measure the mass through the melt or the mass change after melting; #3 record melting points with increasing heating rates; #4 measure the extent of any chemical degradation; #5 analyses for potential isomerisation or polymorphic transformation. To apply this approach, the crystallinity and the purity of the API need to be known before analysis. Thermal analysis has many conventions and sets of stages, for example, the General Stages for Thermal Analysis, Japanese Standards Association (JISK0129:2005, 2005), which maintain standards throughout the field of pharmaceutical and materials science. The novelty of the work reported here is that it addresses an unmet need to optimise the measurement of melting points while acknowledging the potential for partial degradation during the heating cycle.

Experimentally measuring melting points is a relatively straight forward process; MPs may be measured visually using hot stage microscopy or by using glass capillaries mounted in heating blocks (Gaisford et al., 2016), but in order to reduce subjectivity and measure the enthalpy of

melting or fusion (ΔH_f) a calorimeter is typically employed . Differential scanning calorimetry is the most frequently reported form of calorimetry used to record the melting of crystalline materials (Knothe and Dunn, 2009). Only a few milligrams of sample is required, and the DSC measures the endothermic heat flow associated with the first-order phase transition from the crystalline solid state to the liquid state as the temperature is raised (Craig and Royall, 1998; Gaisford et al., 2016). The experimental and theoretical basis of DSC has been covered in depth by many authors. (Craig and Royall, 1998; Feist, 2015; Höhne et al., 2003; Simon, 2001). In a DSC, the differential change in heat flow is measured as a function of temperature, and an endothermic peak is seen during the melting process (figure 1). The onset melting temperature (T_m onset) is used to designate the melting point rather than the peak melting temperature (T_m peak), which is dependent on sample mass and heat transfer across the crystalline particles and the molten liquid; the area of the peak is proportional to the enthalpy of melting ΔH_f (Wunderlich, 1990). Both the determination of T_m and ΔH_f require careful calibration of the DSC using materials of known T_m and ΔH_f; indium, lead and tin are frequently used for such purposes (Gaisford et al., 2016). DSC experimental conditions must be stated and controlled, as a new calibration is required if the heating rate, purge gas, purge gas flow rate, pan type and pan sealing conditions are altered.

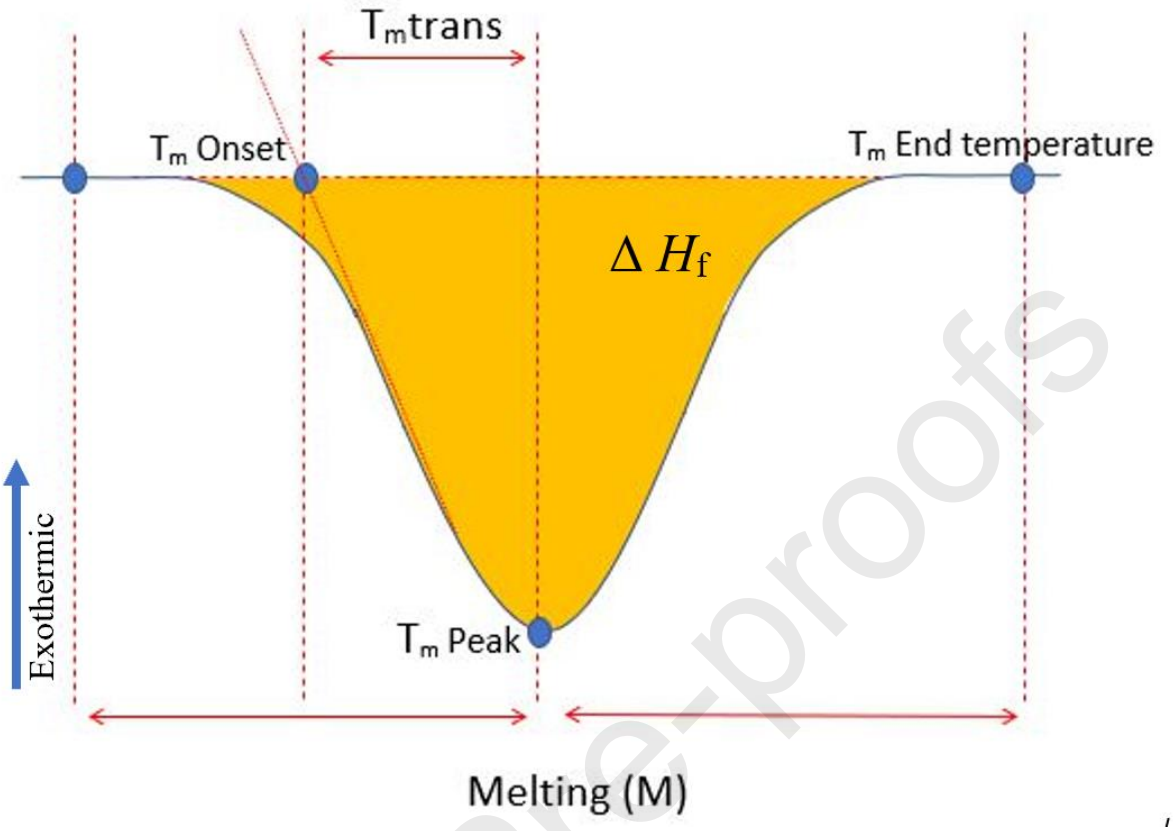


Figure 1: A typical DSC curves representing the endothermic melting peak and its associated parameters (T_m Onset, T_m Peak, T_m End temperature and ΔH_f

DSC experimental conditions need to be optimised to provide accurate melting points; for example, changing heating rates will affect the resolution and sensitivity of the melting peaks. The slower the heating rate, the better the peak resolution, but the weaker the peak sensitivity (Thomas, 2001). The presence of impurities in a crystalline drug substance will lead to melting point depression. This colligative effect may be predicted by a modified form of the van't Hoff equation and used to estimate the amount of the impurity present (Brown, 1979). The purity of such drug substances as acetaminophen, salicylic acid, loratadine and aspirin have been determined using DSC, but in all cases it was assumed that the impurity was no higher than 3% w/w of the sample and that both the impurity and parent drug were stable during heating (Brown, 1979; Candace, 1969; Mathkar et al., 2009). Authors publishing melting points in the pharmaceutical literature and regulatory documentation seldom consider the heat sensitivity of the API and the potential for chemical degradation during the DSC measurement. Pilocarpine HCl is a typical example, the melting point for this drug substance varies considerably in the literature from 188°C to 230°C (Al-handawi et al.,

2014; Cordeiro et al., 2017; Kao et al., 2006; Zoppi et al., 2012). Polymorphism or process-induced disorder was not observed in the pilocarpine HCl samples investigated in the literature, an observation supported by powder x-ray diffraction (Cordeiro et al., 2017). Furthermore, the stated purities of the pilocarpine HCl samples before investigation were high. The origin of the observed variances must, therefore, lie with the level of impurities generated during the DSC experiments, i.e. the drug must have suffered partial degradation within the DSC. Limited thermogravimetric analysis shows a mass loss from solid samples of pilocarpine HCl when heated, but no explanation of what is lost from the samples has, to date, been given (Zoppi et al., 2012). Pilocarpine HCl has two chiral centres and has been observed to suffer epimerization in aqueous solution, converting from pilocarpine (2S:3R) form to isopilocarpine (2R:3R) form. However, such isomerisation has not been observed in the solid-state (Al-handawi et al., 2014). The current authors have an interest in developing new formulations containing the cholinergic agent pilocarpine HCl (Malallah et al., 2018). Thus, a valid and precise value for the melting point of pilocarpine HCl is required to take this work forward.

3 Materials and methods

3.1 X-Ray diffraction

To confirm that the samples under the microscope were crystalline, the powder x-ray diffraction of the as-received pilocarpine HCl was recorded. Powder X-ray diffraction was measured with a Rigaku MiniFlex 600 diffractometer (Rigaku, Tokyo, Japan) operated with Cu Kα radiation (1.5418 Å) at 40 kV and 15 mA, was used to collect patterns in the 2θ range from 3 to 40° at a speed of 2°/min and a step size of 0.01°.

3.2 Optical DSC 450

To visually observe the melting of pilocarpine HCl, (Sigma Aldrich, a lot: #MKBS0848V, ≥98%), an optical DSC 450 manufactured by Linkam was used to record high-resolution images. Samples of pilocarpine HCl, mannitol (Fragon Ltd. Lot RM149/14), and indium (99.99% purity supplied from Perkin Elmer), were placed in open aluminium DSC pans. All pans, n=3 for each sample, were weighed before and after DSC run. Sample weights were close to 3mg, heated from 25°C to 250°C at a heating rate of 10°C/min and observed with a time 5 magnification.

3.3 Measuring the mass pre and post melting using thermogravimetric analysis (TGA)

A thermogravimetric analyser (TA Instruments TGA Q500 V20.13 Build 39) was used to investigate the stability of pilocarpine HCl at elevated temperatures. Sample sizes between 4.6 and 5.1mg, (n=6), were loaded into pre-weighed pin-holed and non-crimped DSC pans which were then loaded onto a platinum TGA pan and heated from room temperature to 150 °C, 215 °C, 230 °C and 250 °C with an isothermal period of 30 minutes using a rate of 10 °C /min. Measurements were carried out under a nitrogen purge with a flow rate of 90 mL/min. The weight loss was recorded in mg as a function of both temperature and time. The pilocarpine HCl from the DSC pans was then dissolved in 100 mL of HPLC water and sonicated for 30 minutes to measure the recovery percentage by high-performance liquid chromatography (HPLC). The DSC pan was then re-weighed to ensure no residual pilocarpine HCl.

3.4 Recording melting point while increasing heating rate

Differential scanning calorimetry studies were performed using a DSC Q20 (TA Instruments, New Castle, DE, USA) with a refrigerated cooling accessory (RCS). The DSC cell was purged with 50 cm^3/min dry nitrogen, and the RCS was purged with 150 cm^3/min nitrogen. The DSC cell was calibrated for temperature and enthalpy for each of the different heating rates following the instrument manufacturer's guidelines for indium (onset melting temperature of 156.6°C), tin (231.9°C) and lead (327.5°C).

A DSC Q20 was used to investigate heating rates at 100°C/min. It was calibrated following the same approach as described above. The universal analysis was used to analyse the DSC curves from both DSCs.

The DSC pans were prepared in two methods, either pin-holed crimped with a hermetic seal or pin-holed non-crimped where the lid was placed on the pan without sealing. For HPLC experiments, the DSC pans (crimped and non-crimped) were re-weighed once the DSC cycle was completed, transferred into HPLC water, the volume of the final solution was made up to 100 mL, and the DSC pans was taken out of the water, left to dry and re-weighed to ensure that the sample had

completely dissolved . The only variation was after finishing the DSC cycle; the non-crimped DSC pan transferred directly into 100 mL of HPLC water while the crimped DSC pan was cut-open and re-weighed to ensure complete recovery of the sample and DSC pan weight after cutting and before dissolving the sample in HPLC water.

To study the effect of different heating rates on the onset temperature, indium, mannitol (purity >=99%) and pilocarpine HCl were loaded into pin-holed crimped DSC pans. The sample size used unless otherwise stated for all DSC experiments was approximately 3mg, with the mass for each experiment recorded accurately on a six-figure balance (Microbalance: Sartorius UK Ltd.). Tested heating rates were 1, 5, 10 and 20°C /min. Experimental conditions followed an equilibration at 25°C for 5 min, ramp to 250°C for all tested samples.

The effect of the DSC pan preparation method on the onset temperature of indium and pilocarpine HCl was then tested using pin-holed crimped with a hermitic seal or pin-holed non-crimped where the lid was placed on the pan without sealing. The sample was heated from 25°C to 250°C using heating rates of 1, 5, 10 and 20°C /min.

The DSC was used to study the effect of different end temperatures on the onset melting temperature of pilocarpine HCl. Following an equilibration at 25°C for 5 min, samples were heated to 150, 215, 230 and 250°C at a rate of 10°C /min using pin-holed crimped DSC pans.

The DSC was then used to study the effect of different pilocarpine HCl weights 1, 5 and 9mg on the onset melting temperature. The experimental condition for these samples followed an equilibration at 25°C for 5 min, ramp to 250°C at a rate of 10°C /min using pin-holed crimped DSC pans.

3.5 Measuring the extent of chemical degradation

3.5.1 High-performance liquid chromatography (HPLC)

Agilent® 1100 Series HPLC was used to separate the degradation products of pilocarpine HCl and to measure the recovery percentage by mass of pilocarpine HCl post-TGA and DSC experiments. The method used a C-18 Gemini- NX 5µm x 4.6 x 250 mm reverse-phase column. The mobile phase consisted of 50:50 % v/v methanol HPLC grade (Fisher Chemicals, BN 1717717) and 0.77 % w/v of 0.1 M ammonium acetate (VWR Chemicals, BN 16I294124, 98% purity) buffered to pH 5.8 using acetic acid (Sigma Aldrich Company, BN SZBD2680V). The pH meter was calibrated for pH 7 using a phosphate buffer solution (Fisher, lot: 1487121) and phthalate buffer (Fisher, lot: 1412687) for pH 4. An injection of 20 µL with needle wash; isocratic flow rate of 1 mL/min at pressure 150 bar, column temperature 37°C and UV analysis with absorbance measured at 229 nm were the analysis specifications. Each sample was injected 6 times, and the average absorbance was calculated with a standard deviation between sample repeats.

3.5.2 Liquid chromatography-mass spectrometry (LC-MS)

The LC-MS analyses were performed on an Agilent 1290 Infinity II LC system fitted with an Agilent 6120 quadruple MSD unit. The analyses were performed on a Monolithic C18 (50 mm x 4.60 mm) column by Phenomenex. The UV detection was performed on the 1290 diode array detector. Mass spectra were registered in both ESI+ and ESI- modes. The running time was 10 minutes, with a volume of injection of 5 µL for each sample. Tested samples were unheated pilocarpine HCl, and pilocarpine HCl samples were heated from 25°C to 100°C and from 25°C to 250°C using heating rate 1°C/min, 20°C/min and 100°C/min, n=3 for each heating rate.

3.5.3 Mohr's titration

A test to investigate the loss of chloride ions (Cl^-) was conducted using Mohr's titration method (Korkmaz, 2001). A standard sodium chloride (NaCl) (Sigma Aldrich Chemicals, BN 52BE0490V, ≥99 % purity) solution was made up by dissolving 1.648 g of NaCl in distilled water, and the solution was made up to the 100mL mark. A standard silver nitrate, $AgNO_3$ (AlfaAesar®, BN 10R002, ≥99.9% purity) solution was prepared by dissolving 4.791 g of $AgNO_3$ in 100 mL distilled water and stored in an amber bottle. Potassium chromate, K_2CrO_4 (Sigma Aldrich Chemicals, BN MKBR5402V, ≥ 99%

purity) indicator was made up by transferring 1.25 g of K_2CrO_4 into a beaker containing 25 mL of distilled water. The solution was left to stand for 12 hours, filtered and made up to 50 mL using HPLC water. For titration, a burette (10 mL) was filled with a 1:10 dilution of the silver nitrate solution. A blank titration was initially carried out using 100 mL of distilled water in a 250 mL conical flask, potassium chromate indicator (1 mL) added to form a light-yellow colour and titrated against the $AgNO_3$ until a brick red colour formed. Two pilocarpine HCl samples were prepared, un-heated pilocarpine HCl was prepared by dissolving 5mg of pilocarpine HCl in 100 mL of HPLC water. The second sample was prepared by dissolving 5.5mg of pilocarpine HCl, heated from room temperature to 215°C by TGA, in a 100 mL of HPLC water. The concentration of chlorine was calculated using titre volumes. The following equations were used to calculate the amount of chloride in a 100 mL sample (Korkmaz, 2001).

$C_{AG^+} x V_{AG^+} = n_{AG^+} = n_{Cl^-}$ Equation (1)

$n_{Cl^-} = m_{Cl^-} / Mw_{Cl^-}$ Equation (2)

Where C is the concentration of Ag^+, V is (titre volume of the analyte - titre volume of the blank), n is the number of moles of Ag^+, m is the mass of Ag^+ and Mw is the molecular weight of Cl^- (35.45 g/mol).

3.5.4 TGA-Mass spectrometry (TGA-MS)

TGA can provide quantitative information on mass loss from the sample, and the MS was used to provide information on the nature of the volatile products that were lost from the sample. Therefore, this combined system was used for the stability assessment of pilocarpine HCl. A pilocarpine HCl sample, 30mg, was run on a Perkin Elmer Pyris 1 TGA coupled to a Hiden HPR 20 ms unit, and the carrier gas used was CP Grade helium at 30 mL/minute. Scans for chloride isotopes Cl^{35} and Cl^{37} using multiple ion detection were carried out.

3.6 Monitoring the potential of isomerisation using polarimeter

An AD440+ polarimeter equipped with a sodium lamp (λ=589) was used with a proprietary 20cm sample cell. This instrument used to study the isomerisation of pilocarpine HCl to isopilocarpine. The polarimeter was prepared for calibration by zeroing the instrument with an empty cell compartment and then calibrated with a sucrose solution (26g/100mL HPLC water, n=3) following the manufacturer's recommendations (Supplementary 9.1). A lactose sample was prepared and tested in the instrument for further validation (Jawad et al., 2014). The stability of unheated pilocarpine HCl in methanol and water was tested over 48h for the determination of the suitable solvent for the isomerisation study (20mg/20mL of water or methanol HPLC grade, n=6), supplementary 9.1.

Isopilocarpine was obtained from pilocarpine HCl by racemisation. The 1 mL of 0.1 M NaOH was added to a 5 mL solution of 0.5mg/mL pilocarpine HCl in a 25 mL volumetric flask to allow deprotonation. After 3 hours, 1 mL 0.1 M HCl then added to allow reprotonation. The volume of the final resulting solution was completed to 25 mL by sample diluents (El Deeb et al., 2006). The isomerisation/epimerization of pilocarpine HCl has been tested by heating pilocarpine HCl by DSC. Pilocarpine HCl samples were heated from 25°C to 100°C and from 25°C to 250°C using heating rate 1°C/min, 20°C/min and 100°C/min, n=3 for each heating rate. All Samples were loaded in pin-holed non-cramped DSC pans. Heated pilocarpine HCl samples then dissolved in 20mL of HPLC water for further characterisation using HPLC, LC-MS and polarimeter. Unheated pilocarpine HCl samples were also prepared as a control, n=3. The specific rotation was calculated using the equation below at 23°C ±0.5°C.

$$[\alpha]_{\lambda}^{T} = \frac{100 * \alpha}{\iota * c} \qquad \textit{Equation 3}$$

Where α is the measured rotation in degrees. ι is the path length in decimeters and C is the concentration of the liquid in g/mL for a sample at a temperature of T and wavelength λ in nanometers.

3.7 Statistical analysis

For results analysis and calculations of significant differences in this paper, Prism 7 stats program was used. Un-paired T-test was carried where the P-value represents the level of significance, which is significant at the 0.05% confidence level. Significantly different ($P < 0.05$).

4 Results

4.1 Chemical and Physical purity of the as-received pilocarpine HCl.

To confirm the crystallinity of pilocarpine HCl powder, a diffractogram of the sample was recorded, with the diffraction patterns obtained (Supplementary 9.2). The diffraction pattern included peaks at diffraction angles, 2θ: 18.06°, 20.35°, 21.71°, 25.33°, indicating that the drug was present in a crystalline form and contained a single polymorph (Zoppi et al., 2012). HPLC confirmed the as received pilocarpine HCl was highly pure with a purity of greater than 98% and a chromatogram identical to those recorded by many previous studies, for example, Bundgaard & Hansen (1982). The linear HPLC calibration graph (r^2 of 0.999), gave limits of detection (LOD) and limits of quantification (LOQ) for pilocarpine HCl of 3.7 μg/mL and 12.4 μg/mL, respectively.

4.2 Stage #1: Visual Observation

The melting of indium, mannitol and pilocarpine HCl were visualised using an optical DSC 450 instrument (Figure 2). Indium and mannitol were investigated because of their reported stability and lack of any degradation during their melting so they could act as controls, (Gabbott, 2008; Gabbott et al., 2003) The micrographs for indium and mannitol showed only melting without any indication of thermal degradation, i.e. there was no discolouration or visible emission of vapour. Indium is a soft, stable and highly pure metal commonly used for the calibration of differential calorimeters; its melting point of 156°C is observed in the DSC by a narrow endothermic melting peak (Gabbott, 2008). Because of the viscosity and optical properties of the molten metal, indium's melting is difficult to observe visually under a microscope. For mannitol, this is not the case as it can be seen that as the melting point is reached and surpassed, the crystals soften, and the molten sugar begins to flow. Thus, when visually observing melting, using capillary or microscope-based methods, metallic calibration materials, as demonstrated by indium, may not be the optimal choice, because of the difficulty in visualising the melting and phase change for these materials, Pure and stable organic crystalline solids are potentially a better choice for calibration samples. For pilocarpine HCl, unlike for indium and mannitol, a change in colour appeared just after melting, which indicated potential thermal degradation within the sample (Figure 2). Thus, further characterisation of the thermal stability of pilocarpine HCl was warranted.

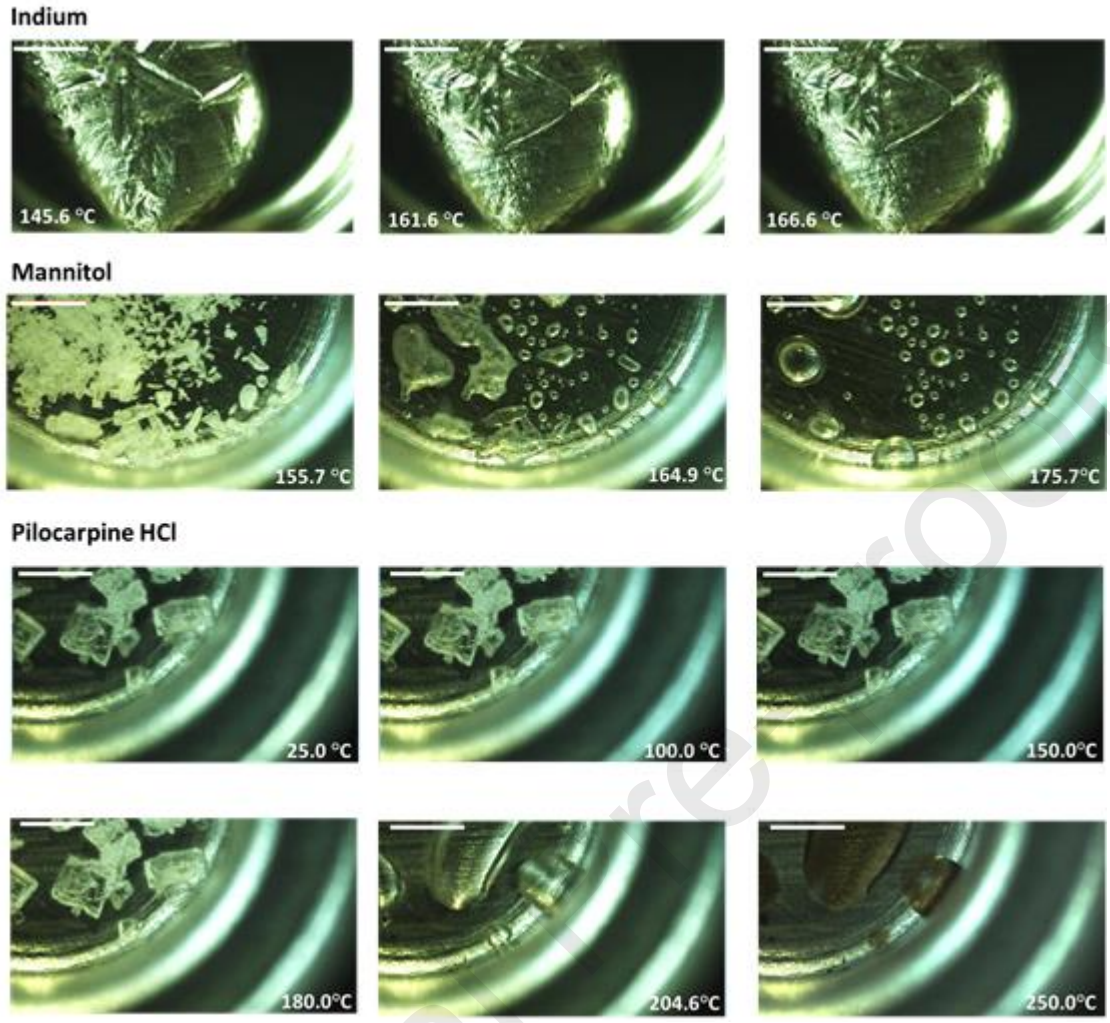


Figure 2: Crystal images of indium, mannitol and pilocarpine HCl before melting, partially melt and complete melt using a heating rate of 10℃/min and magnification of 5X. Scale bars represents 700µm.

4.3 Stage #2: Measurement of mass

TGA was used to quantify any mass loss from samples of pilocarpine during heating. Samples were placed into the TGA pre-loaded in pin-holed DSC sample pans in order to mimic the conditions during the melting point measurements. Any volatile material lost as a consequence of thermal degradation during heating could escape through the pinhole; such an approach is commonly used in pharmaceutical thermal analysis (Gabbott 2008). The samples tested in the TGA were recovered and subjected to analysis by HPLC; both techniques indicated that below its melting point, pilocarpine HCl lost only a minimal amount of volatile material. For example, as the temperature was increased from room temperature to 150 °C mass loss was very low, 2% $\pm$ 0.5 (w/w). The method of extraction of the sample from the pans used in the TGA experiments was validated by measuring the % recovery of pilocarpine HCl from unheated pans, the amount recovered was within experimental error of the as-received pilocarpine HCl with a purity of 98% (figure 3). For those experiments where the holding temperature was above the melting point of pilocarpine HCl a significant loss in mass was observed, as well as a dramatic loss in the drug content recovered, figure 3 (I). When pilocarpine HCl was held just above its melting point, close to 215°C for 30minutes, only 53 ± 1.5% (w/w) of pilocarpine HCl was recovered from the pan. Results from TGA and HPLC were in line, as the holding temperature increased both the mass and drug content recovered decreased, ending with 0% (w/w) drug content recovered when the temperature was set to 250°C, figure 3 (I). The measurement of mass loss indicated the occurrence of thermal degradation, a small loss below the melting point, which increased dramatically at and post melting. Investigating the sample post TGA with a drug content indicating HPLC assay confirmed pilocarpine HCl as a heat-sensitive drug, but further chemical analysis was required to characterise the origin of the mass loss, see section 4.5.

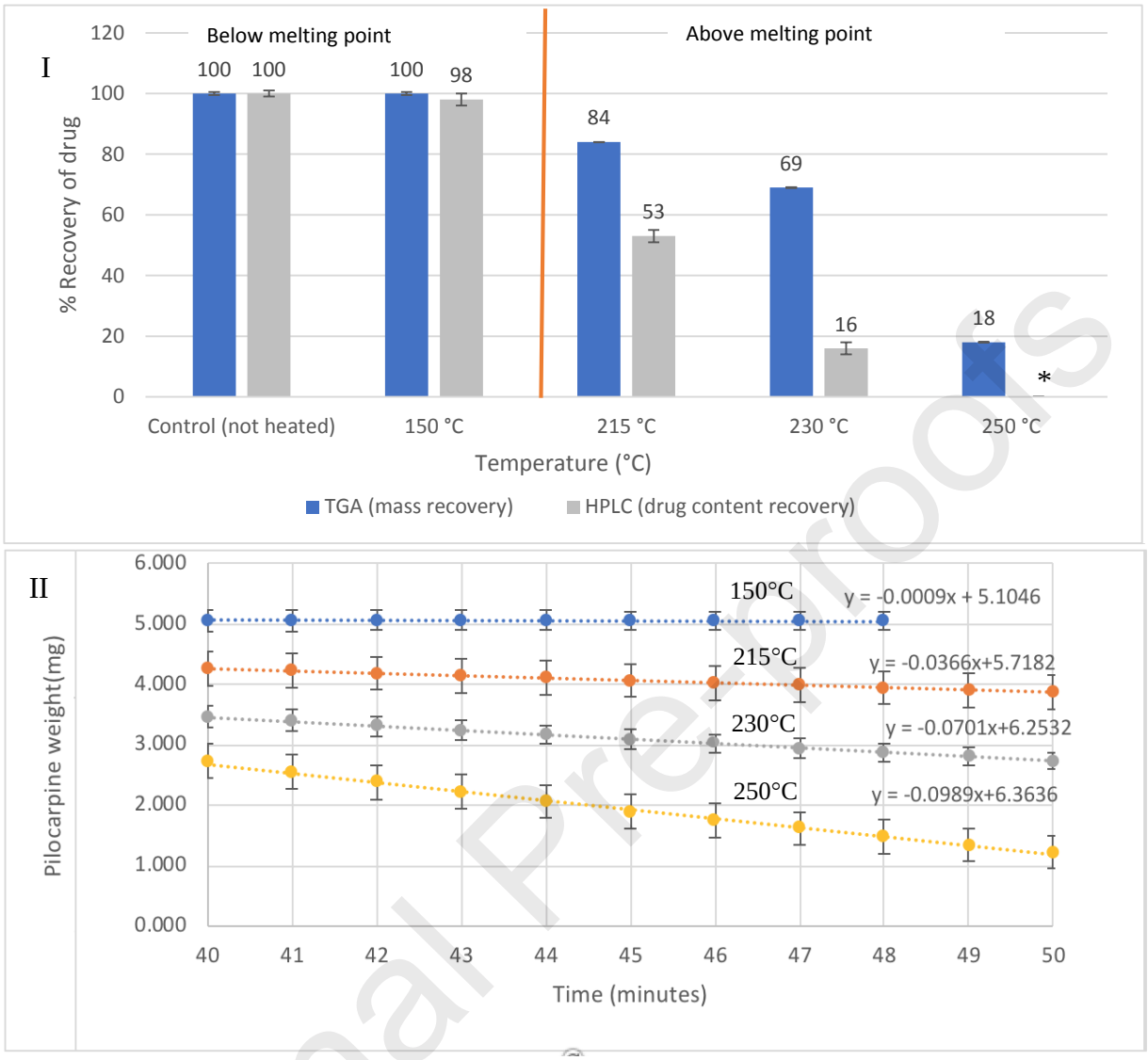


*Figure 3 (I) Effect of different final temperatures on the percentage mass recovery by TGA and HPLC. Pilocarpine HCl sample weight varied between 4.6 and 5.1mg loaded into a pin-holed non-crimped DSC pan that was placed onto a platinum TGA pan. Samples were heated from 25 ℃ up to 150 ℃, 215 ℃, 230 ℃ and 250 ℃ at a heating rate of 10 ℃/min and this was followed by 30 minutes isothermal period after the stated temperature was achieved. A number of repeats (n=6). Error bars represent the standard deviation between sample repeats. * symbol indicates that no pilocarpine HCl was recovered by HPLC. (II) Shows TGA curves of pilocarpine HCl and the mass loss once the holding temperature had been achieved. The time represents both the heating and the isothermal part of the experiment. Error bars represent the standard deviation between sample repeats. R^2 ranges close to 0.998.*

4.4 Stage #3 Melting point measurement as a function of heating rate

Application of stages #1 & #2 clearly showed that pilocarpine HCl suffers from thermal degradation. Thus, when measuring the melting point of this drug using differential scanning calorimetry, it can be hypothesised that the speed of heating will influence the onset of melting. The faster the measurement, the less time available to generate new impurities, and so any lowering of the melting point caused by the presence of degradation products will be reduced. The investigation of stage #3 with pilocarpine HCl required additional validation using thermally stable crystalline materials to take account of any instrumental effects on the observed melting onsets. Indium and mannitol performed this role in the present study, as their melting has been previously studied as a function of heating rate, and they are regarded as stable throughout their (Liu et al., 2006; Pijpers et al., 2002; Price, 1995; Saunders et al., 2004). The impact of different heating rates, 1°C/min, 5°C/min, 10°C/min and 20°C/min on the melting points for all three materials is shown in figure 4, 5 and 6. The DSC curves of the three materials are presented in figure 4.

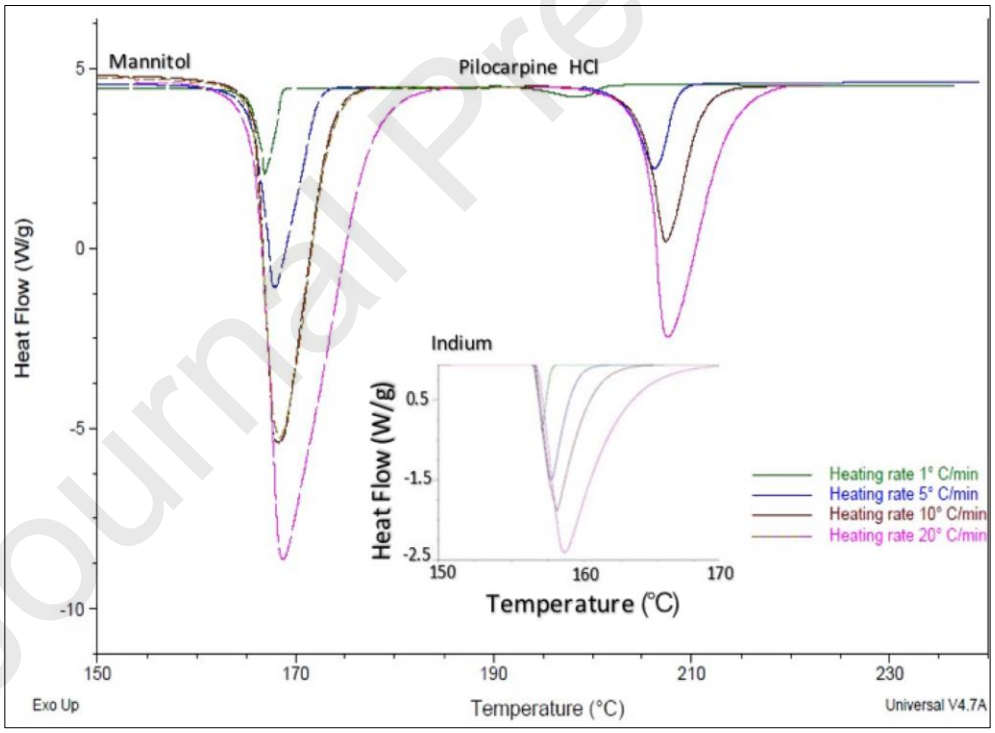
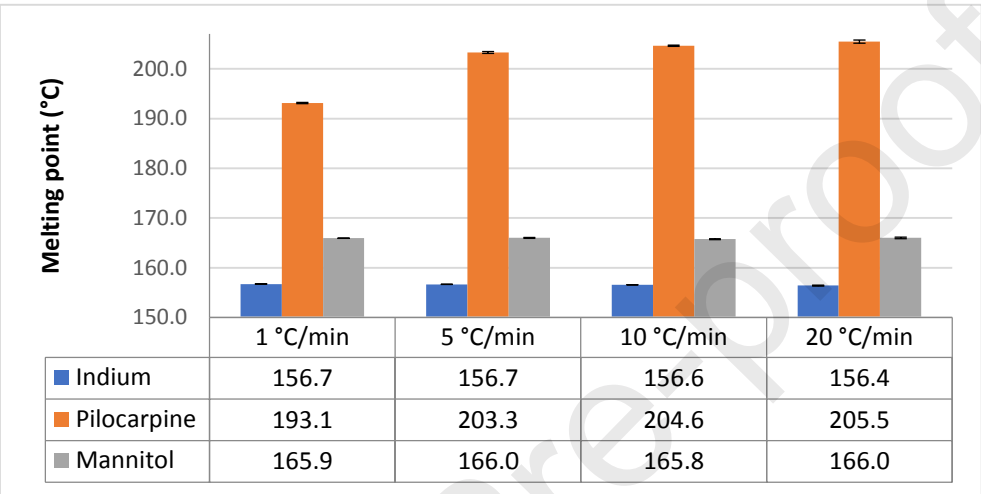


Figure 4: DSC curves to illustrates the effect of different heating rates on the melting point of indium, mannitol and pilocarpine HCl samples. Accurately weighed samples, (close to 3mg) were loaded into pin-holed crimped DSC pans, and heated from room temperature to 250 ℃.

As expected, for both indium and mannitol, no significant differences between the onset melting temperature with heating rates of 1°C/min to 20°C/min were recorded (p-values were 0.8594 and 0.7101, respectively), figure 5. The DSC was calibrated at these different heating rates, so instrumental effects were minimised as seen by the lack of variability in the melting points for indium and mannitol as a function of heating rate.



	1 °C/min	5 °C/min	10 °C/min	20 °C/min
Indium	156.7	156.7	156.6	156.4
Pilocarpine	193.1	203.3	204.6	205.5
Mannitol	165.9	166.0	165.8	166.0

Figure 5: shows melting point values of indium, mannitol and pilocarpine HCl using different heating rates. N=3, error bard represents the standard deviation between the sample repeats.

The melting points for indium and mannitol are comparable to the literature values for their melting points at all heating rates. For pilocarpine HCl, the value of onset melting temperature started to increase significantly with increasing the heating rate, with an onset temperature of 192.05 ± 0.32°C & 205.5 ± 0.34°C for heating rates 1°C/min & 20°C/min respectively (p-value <0.0001), figure 5.

The effect of DSC pan preparation on the onset of melting temperature of indium and pilocarpine HCl was studied using pin-holed/crimped and non-crimped DSC pans, figure 6, and more detailed information is provided in supplementary 9.3. Figure 6 shows that the onset temperature of indium does not change with pin-holed crimped and non-crimped pin-holed DSC pans. There is a significant difference in the onset melting temperature of pilocarpine HCl between crimped and non-crimped DSC pans at each heating rate (p-value was 0.0004 and 0.1135 for heating rate 1 and 20, respectively), figure 6. Interestingly, with all heating rates, from 1 °C /min to 20°C /min, crimped

DSC pans of pilocarpine HCl showed lower onset temperatures in comparison to the non-crimped DSC pans. Also, the mass loss of pilocarpine HCl from the pin-holed crimped and non-crimped DSC pans was different. For example, with heating rate 1 °C /min, the mass loss was 1.20 ± 0.20mg with non-crimped pans and 0.42 ± 0.01mg with crimped DSC pans.

The thermal degradation identified by the application of stages #1 & #2 had a significant impact on the melting point of pilocarpine. Applying stage #3 showed that it is the effect is mitigated by increasing the heating rate. Additional experiments with a heating rate of 100°C/min gave an onset of melting equal to 206.9 ± 0.4°C which is very close to the melting point determined at 20°C/min, the onset melting points for both heating rates are not significantly different (P-value 0.2863). At 100°C/min, the amount of drug recovered by HPLC after melting was similar to the starting value of 98 ±1. The impact of the type of lid used and the loss of considerable amounts of material at more conventional heating rates warranted the determination of the extent of chemical degradation and a characterisation of what is lost from the pilocarpine HCl powder during its melt.

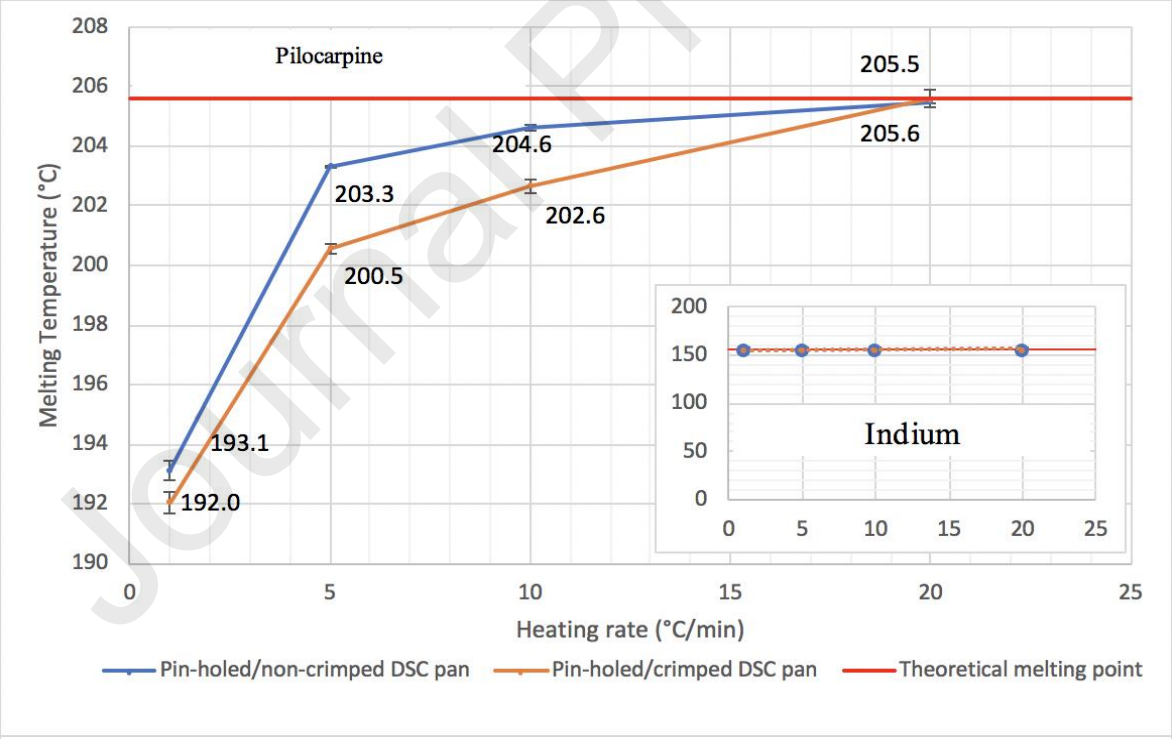


Figure 6: Effect of varying heating rates and DSC pans preparation (crimped and non-crimped DSC pans) on the measured onset temperature of indium and pilocarpine HCl. Number of repeats (n=3) for each point. All samples were heated from room temperature to 250°C. Each sample were injected 6 times in the HPLC. The illustrated results represent the mean of six values with the error bars determine from the standard deviation between the sample repeats.

4.5 Stage #4 Testing the extent of chemical degradation.

Mohr's titration method was used to determine the percentage of chloride ions in pilocarpine HCl (as received), and the pilocarpine HCl when heated to 215 °C within TGA while enclosed in a crimped pin-holed DSC pan. HPLC grade water was used as a blank, and NaCl was used to validate the titration method, (table 1). The solution containing pure pilocarpine HCl and potassium chromate appeared clear yellow. Halfway through the titration, with an addition of silver nitrate, the solution turned cloudy yellow, and the endpoint of titration was as indicated by a red brick precipitate.

Table 1: Determination of chloride ions in pilocarpine HCl by titration. The mean volume of samples and the concentration of silver nitrate ($AgNO_3$) were constant, 100 mL and 2.82x10^{-5} moles. mL^{-1}, respectively. Accurately weighed samples (4 to 6mg) of pilocarpine HCl were loaded into pin-holed non-crimped DSC pans and heated from 25°C to 215°C with isothermal for 30 minutes using a heating rate of 10°C/min in the TGA. The number of repeats (n=3), the error bars represents the standard deviation.

Sample name	Mean volume of $AgNO_3$ (mL)	Mean amount of Cl ions detected in 100 mL sample (mg)	% Cl ions
Blank/ HPLC grade water	1.68 ± 0.23	-	0%
NaCl	2.08 ± 0.05	0.400 ± 0.02	100 %
Non-heated pilocarpine HCl	2.08 ± 0.03	0.400 ± 0.05	100%
Heated pilocarpine HCl (215 °C)	1.98 ± 0.02	0.299 ± 0.02	60 %

The number of chloride ions per 100 mL decreased in the pilocarpine HCl sample heated to 215 °C. The mean chloride content expected for pure NaCl and pure pilocarpine HCl was 0.721 ± 0.043mg and 0.646 ± 0.050mg, respectively. The mean chloride content was calculated using the equation below:

Equation 4:

$$\frac{Molecular\ wt.\ Cl^{-}}{Molecular\ wt.\ (NaCl\ or\ drug) \times Amount\ (mg)\ disolved\ in\ 100\ mL}$$

From the titration, pure pilocarpine HCl, for example, 4.463mg, contained 0.400mg of chloride ions. Heated pilocarpine with a mass of 5.585mg, should, therefore, have a chloride ion value of 0.500mg. However, only 0.299mg were detected in titration, accounting for 60 % of the expected chloride

ion content. Therefore, the loss of the chloride must be associated with the evaporation from the DSC pan of a volatile chloride as a consequence of degradation. A simple hypothesis to explain the titration results is that the disproportionation of HCl causes this chloride loss from the weak base pilocarpine at elevated temperatures.

To test the hypothesis that HCl is being lost from pilocarpine HCl when heated to and above its melting point, TGA coupled with mass spectrometry was used to investigate the composition of the evolved gas. Within the TGA curves, figure 7, the TGA of pilocarpine HCl (red line) showed a gradual mass loss starting close to 193°C. A sharp decrease in the mass of pilocarpine HCl samples was recorded just after its melting point, around 200 °C. A strong association was recorded between the sample loss in the TGA and MS detection of Cl^{35} (blue dotted line) over the period, from 16 to 22 min, figure 7. The results clearly show that the mass loss form the DSC pan is associated with evaporation of HCl^{35}.

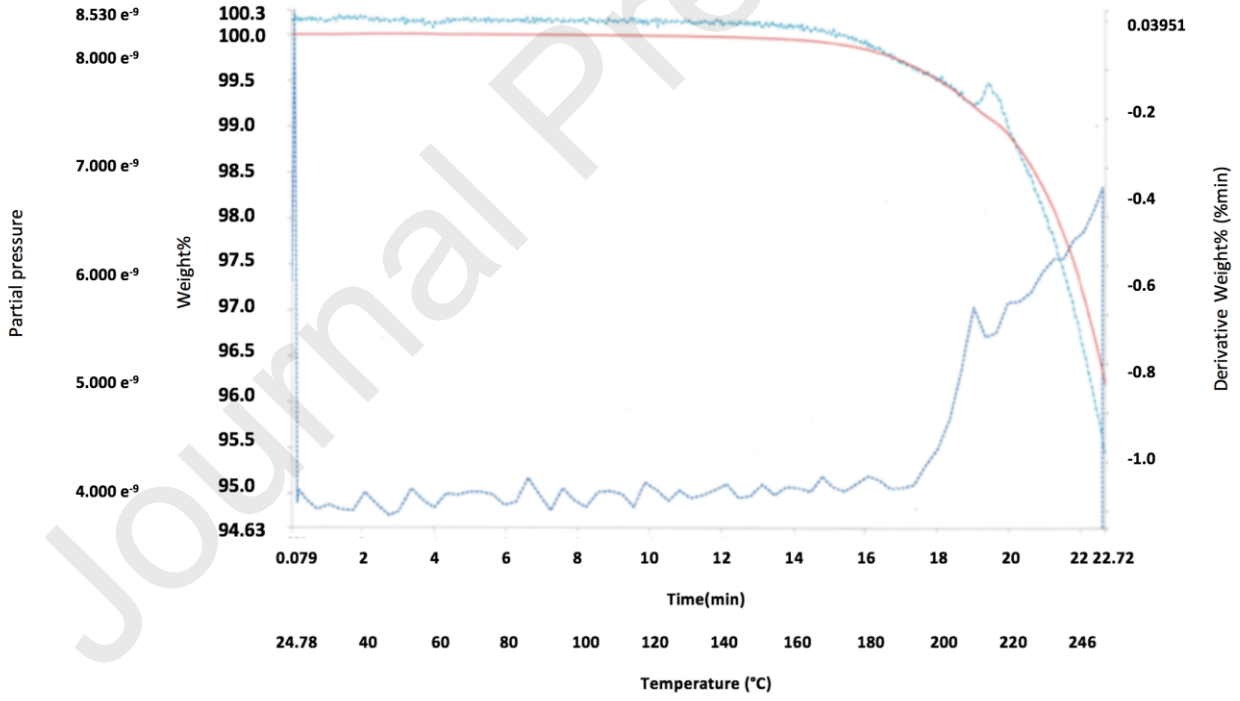


Figure 7: TGA curves *(red colour)-MS spectrum (blue colour) of 30mg pilocarpine HCl, heated from 25 ℃ to 250 ℃ using a heating rate of 10 ℃/min. The partial pressure is proportional to the concentration of the hydrogen chloride isotope 35. MS instrument was set to detect hydrogen chloride gas containing isotope* Cl^{35}.

The effect of different heating rates and DSC pan preparation method on the recovery percentage of preheated pilocarpine HCl is illustrated in figure 5 and 6. The HPLC reference was non-heated pilocarpine HCl dissolved in HPLC water grade. Pilocarpine HCl samples were loaded into pin-holed crimped, or non-crimped DSC pans with a weight of 3mg, heated from 25°C to 250°C, using heating rates of 1, 5, 10 and 20°C /min. In general, the recovery percentage of pilocarpine HCl increases with higher heating rates. For example, with a heating rate of 1°C/min, the recovery percentages were 7 $\pm$ 0.2 % and 31 $\pm$ 1% for crimped and non-crimped DSC pans, respectively. While with a heating rate of 20°C/min, the recovery percentages of the drug were above 90%, for both crimped and non-crimped DSC pans.

Interestingly, there is a significant difference (p-value of 0.0004 and <0.0001) in drug recovery percentage between crimped and non-crimped DSC pans using heating rates 1°C/min and 5°C/min, respectively. With the heating rate of 1°C/min, only 7% of the pilocarpine HCl was recovered from crimped DSC pans in comparison to 31% recovery from non-crimped DSC pans. A similar trend was noticed with a heating rate of 5°C/min, where the percentage recovery from crimped DSC pans was lower than non-crimped DSC pans, 38 % and 80%, respectively, figure 8. The low percentage recovery of pilocarpine HCl was always combined with the appearance of 3 new peaks in the HPLC chromatograms, suggesting many different types of degradation products.

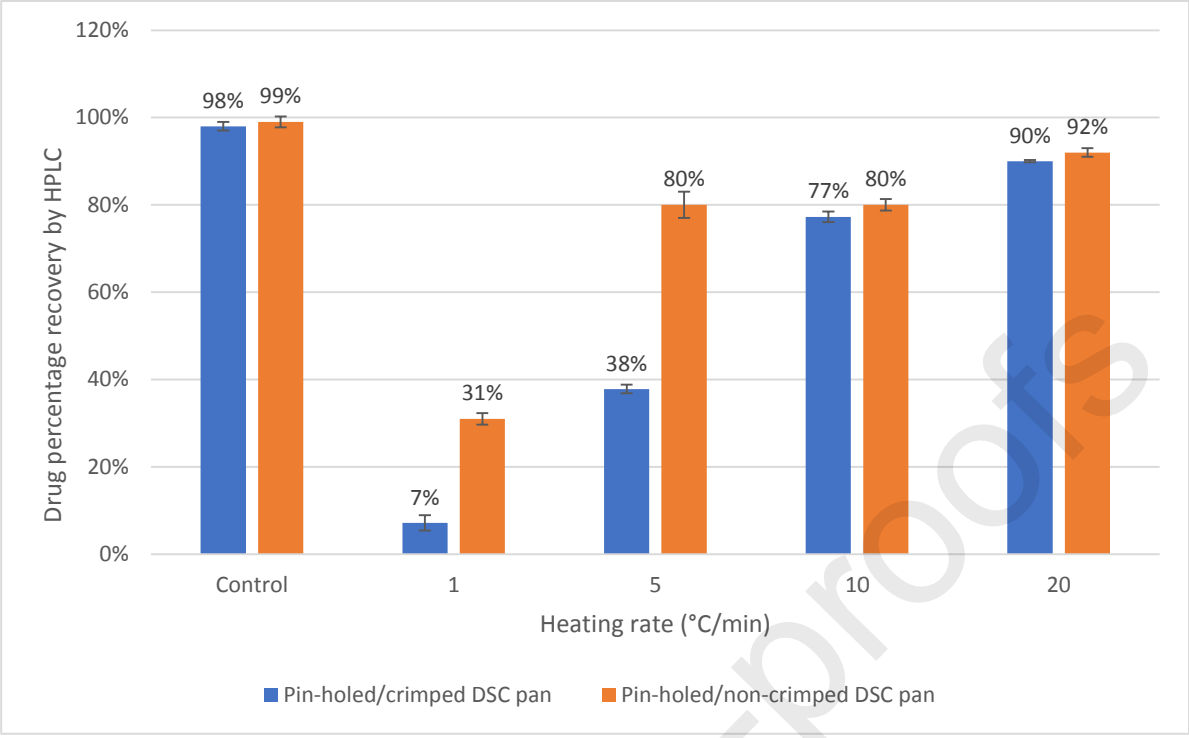


Figure 8: Effect of varying heating rates and DSC pans preparation (crimped and non-crimped DSC pans) on the recovery percentages of pilocarpine HCl post DSC (II). Number of repeats (n=3) for each point. All samples were heated from room temperature to 250°C. Each sample were injected 6 times in the HPLC. The illustrated results represent the mean of six values with the error bars determine from the standard deviation between the sample repeats.

Pilocarpine HCl samples were heated from room temperature to 150, 215, 230 and 250°C at a heating rate of 10°C/min, (supplementary 9.6). There was no significant difference between the onset melting temperature; for example, a p-value of 0.7101 was observed when the recorded values at 215°C and 250°C were compared. The effect of heating and immediately cooling, i.e. without an isothermal period, and with the inclusion of a 30 minutes isothermal period on the recovery percentage of pilocarpine HCl was significant (p-value < 0.0001, figure 9 and 3). More pilocarpine HCl was recovered when the samples were heated and merely cooled. However, to measure the enthalpy of fusion, an end temperature of at least 230°C and ideally 250°C is required. For both end temperatures, a significant amount of pilocarpine HCl as compared to the non-heated samples had degraded.

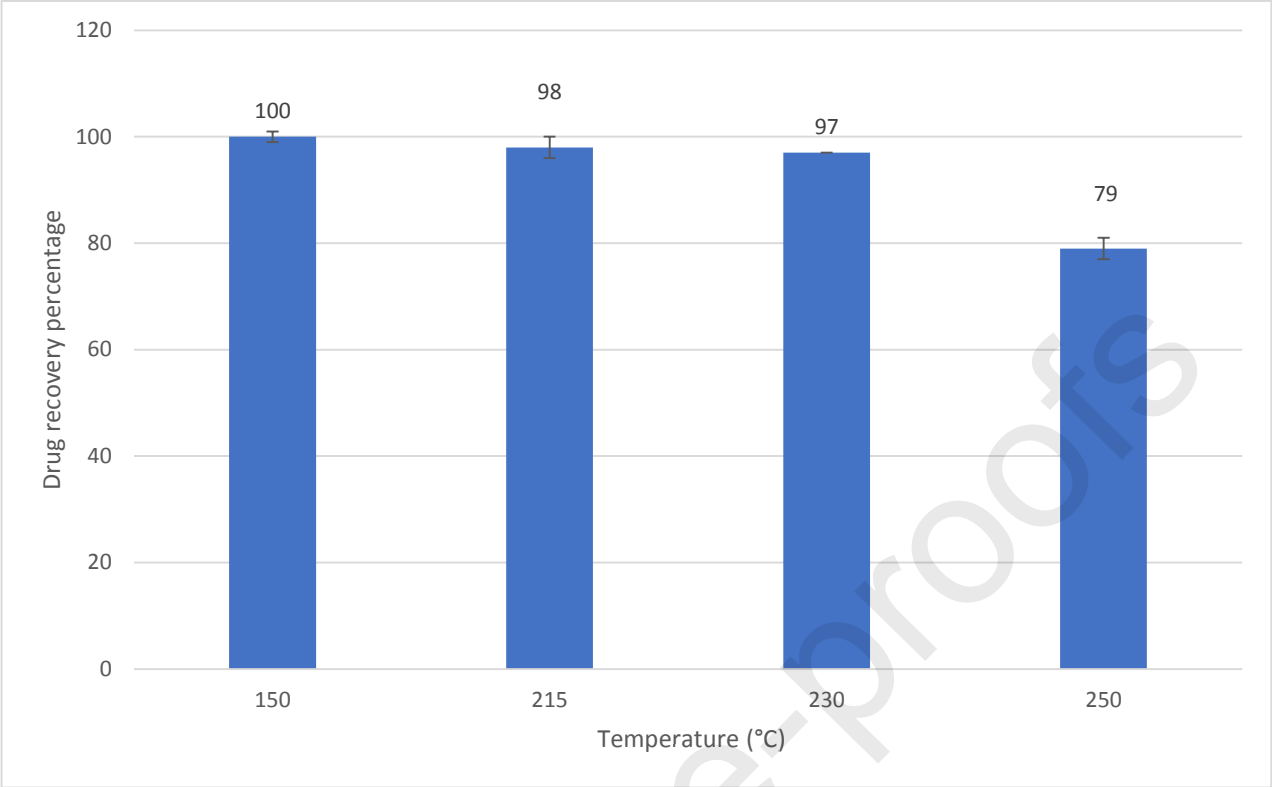


Figure 9: The effect of varying target temperatures on the recovery percentage of pilocarpine HCl. The sample weights were approximately 3mg and the number of tested samples (n=3) for each target temperature. All samples were heated using a heating rate of 10°C/min and immediately cooled back to room temperature without an isothermal period. Each sample was injected 6 times in the HPLC. The illustrated results represent the average recovery value for each heating rate and error bars that represent the standard deviation between samples repeats

4.6 Stage #5 Analysis for potential isomerisation.

Many drug substances show stereoisomerism; pilocarpine HCl follows this observation as it has two chiral centres at position 3 and 4 on its substituted furanone ring. In aqueous solution, the 3S, 4R form of pilocarpine will epimerize directly to the 3R, 4R form otherwise known as isopilocarpine, and the rate of this reaction increases with temperature (Bundgaard & Hansen 1982). Thus, polarimetry was used to investigate the possibility of epimerization occurring during heating of crystalline pilocarpine HCl powder. In order to interpret the results, a sample of isopilocarpine was prepared to act as a control for the specific rotation experiments. The preparation method followed a frequently cited literature method (El Deeb et al., 2006), and it can be seen from figure 10 that it was possible to separate isopilocarpine from pilocarpine by conventional reverse-phase HPLC, this is not uncommon for diastereoisomers (El Deeb et al., 2006).

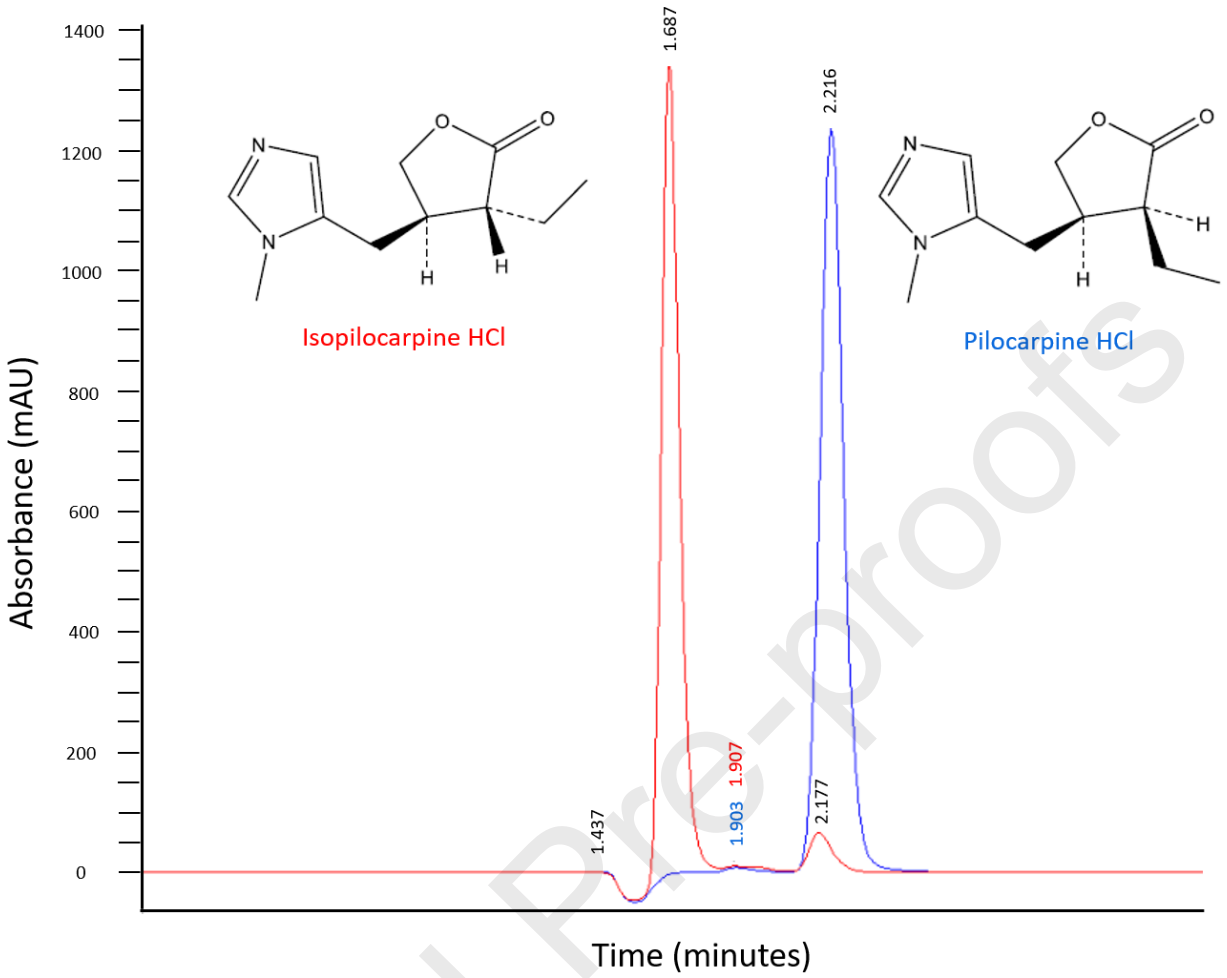


Figure 10: HPLC chromatograms of pilocarpine HCl and in vitro isopilocarpine. The blue coloured peak represents the unheated pilocarpine HCl dissolved in HPLC water, showing two peaks at 1.9 minutes and 2.2 minutes. The red coloured peak represents the in vitro prepared isopilocarpine, showing three peaks at 1.6 minutes, 1.9 minutes and 2.1 minutes. N=3 for each sample and the number of injections was 6.

The extinction coefficients for both forms of pilocarpine are expected to be close, and thus the size of an earlier eluting peak for iso pilocarpine and the near absence of the peak for pilocarpine indicates a near-complete conversion of pilocarpine to isopilocarpine in the material under investigation, figure 10. The specific rotation results are illustrated in table 2. The calibration of the polarimeter instrument was further confirmed by measuring the specific rotation of lactose sample. The specific rotation of 84.9± 1.21 for lactose was similar to the results reported in the study by Jawad et al. (2014).

Table 2: illustrate isomerisation of pilocarpine HCl using DSC, HPLC, LC-MS and polarimeter. Unheated pilocarpine HCl samples were preprepared as control, n=3. In vitro prepared Isopilocarpine was prepared in the lab,n=3. Three samples (for each heating rate) of pilocarpine HCl were heated from 25°C to 100°C using heating rate 1°C/min, 20°C/min and 100°C/min DSC,n=3. Pilocarpine HCl was heated from 25°C to 250°C using heating rate 1°C/min, 20°C/min. Each sample then dissolved in 20mL of HPLC water and characterised using different instruments. All of the LC-MS peaks showed (m/z) calculated $[M+H]^+$=209.1. The ± symbol represents the standard deviation between samples repeats.

Sample Name	**The percentage recovery percentage of pilocarpine HCl by HPLC**	**Number of peaks / (m/z) of signals calculated $[M+H]^+$**	**Specific rotation**
Unheated Pilocarpine HCl	98± 10	One peak /209.1	85.40 ± 0.85
In vitro prepared Isopilocarpine	3± 1.60	One peak/209.1	-15.56 ± 1.75
Pilocarpine HCl 100°C – HR1°C/min	95±1.10	First peak/209.1 Second peak/209.1	0.00 ± 0.10 (racemic mixture)
Pilocarpine HCl 100°C – HR20°C/min	98± 13	First peak/209.1 Second peak/209.1	33.38 ± 8.40
Pilocarpine HCl 100°C – HR100°C/min	98± 16	One peak/209.1	83.70 ± 0.25
Pilocarpine HCl 250°C – HR1◦C/min	31±10	Multiple peaks between retention time of 1.5 to 2 mins	10.73 ± 13
Pilocarpine HCl 250°C – HR20°C/min	92±10	First peak/209.1 Second peak/209.1	33.16 ±11.3
Pilocarpine HCl 250°C – HR100°C/min	98±10	One peak/209.1	69.20 ± 8.99

The unheated pilocarpine HCl showed 2 peaks in HPLC. The small first peak with an area under the curve (AUC) of 47, originated from the 2% impurity reported by the supplier, and this peak was detected in all of the analysed samples. The second peak showed a very much AUC the curve equal to 6360, which is related to pilocarpine HCl, where (98%± 1%) of the pilocarpine HCl was recovered.

The HPLC chromatography indicated that the pilocarpine HCl heated from 25°C to 250°C with heating rate 20°C/min was isomerised to produce the isopilocarpine along with the reduction of the recovery percentage of the pilocarpine HCl. However, with pilocarpine HCl heated from 25°C to 250°C using a heating rate of 1°C/min, multiple peaks were appeared indicating the isomerisation and thermal degradation of the API. The *In-vitro* prepared Isopilocarpine results supported the data given in the table above.

Separate LC-MS experiments showed a peak for the pilocarpine HCl, and a peak for the prepared sample of isopilocarpine, (similar to the findings from the conventional HPCL), these peaks had different retention times but the same molecular weight for the molecular ion. For the heated pilocarpine HCl samples, the appearance of more than one peak with the same (m/z) calculated $[M+H]^{+}$=209.1, indicated that epimerisation had occurred within pilocarpine HCl, the extent of epimerisation was influenced by heating rates and different end temperatures as shown in table 2. The observation of epimerisation was further supported by the change in the observed specific rotation for heated samples, (table 2). For example, the specific rotation for the unheated pilocarpine HCl dropped from 85.40 ± 0.85 to zero when pilocarpine HCl heated from 25°C to 100°C using heating rate 1°C/min. Based on the previous observations and the results from table 2, it is evident that heating rate affects the degree of epimerisation and thus the isomeric composition of pilocarpine HCl. However, the faster the heating rate, the more of the original and active stereoisomer of pilocarpine HCl is recovered.

5 Discussion

The assumption that a crystalline material remains chemically and thermally stable during the measurement of its melting point is rarely challenged. The five stage approach proposed here provides a framework to validate the measurement of a melting point if the sensitivity to heat is suspected. Applying this approach to pilocarpine HCl has provided new information concerning disproportion of the salt and its isomerisation within the DSC pan, and with this knowledge, more effective heating cycles may be designed to minimise the impact of these processes on the variance observed when measuring melting points.

Measuring the melting point of a crystalline drug using a DSC operating at only a single heating rate is not adequate, additional experiments are required. Visual observation is the first stage that should be applied, and in the case of pilocarpine HCl, the first indication of thermal sensitivity was the discolouration of the molten material. Stable materials, for example, mannitol and indium, tend not to show changes in colour. Identifying melting using visual inspection for metals like indium is not recommended because the optical properties of metallic materials do not dramatically change through their melt. Visual observation will not be useful if thermal degradation produces non-coloured products, so the further quantitative analysis is required. Nevertheless, thermal analytical approaches benefit from an inspection of the sample post melting; observation of colour changes indicates potential instability. If a form of hot-stage microscopy is not available, inspecting the pan before and after heating is a simple compromise.

The second stage, the measurement of mass may be used as a simple method of predicting the stability of a heat-sensitive drug if it degrades with the loss of volatile products. For pilocarpine HCl, a zero-order model was used to fit the degradation at a series of holding temperatures to establish the activation energy and shelve life of pilocarpine HCl, figure 3 (II). An Arrhenius plot of pilocarpine HCl was constructed using the rate constants measured at the four different temperatures. The regression coefficient (r^2) of the line was 0.991 and with an activation energy of 98.3 kJ/mol. Extrapolating the line to 25°C enabled an estimation of the shelve life of pilocarpine HCl at room

temperature, assuming 5 % decomposition, and this yielded a shelve life of approximately 6.6 years, which is close to the supplier's (Sigma-Aldrich) stated shelve life of 5 years.

The estimated activation energy most probably has contributions from the disproportionation of the HCl salt, and then vaporisation of HCl and in addition to the further decomposition of pilocarpine, as the heat of vaporisation of hydrogen chloride is within the range from 14.8 to 17 kJ/mol (GiauquB & Wiebe, 1928). Thus, the application of the second stage is essential for the estimation of decomposition kinetics.

Applying stage 3, i.e. recording the impact of a variation in the heating rate is vital for all processes observed by differential scanning calorimetry not just for the measurement of melting points. Changing heating rates affects the resolution and sensitivity of the generated DSC peaks. The faster the heating rate, the higher the peak sensitivity but, the weaker the peak resolution. (Thomas 2001). However, in terms of melting point measurement if the DSC has been appropriately calibrated, then changing the heating rate should not affect the onset of melting (Gabbott 2008). A change in the onset of melting for pilocarpine HCl was observed because different amounts of degradation products were formed at the different heating rates, which lowered the melting point to different degrees. Only by heating relatively quickly was this variation in melting points diminished.

In order to examine what was evolved and the purity of the pilocarpine which remained combinations of pinholes, crimping and non-crimping had to be applied to the DSC pan lids. Crimped DSC pans are standard for the analysis of pharmaceutical samples. However, the non-crimped DSC pans were used in this study for the ease of further chemical analysis post-DSC experiments. Different end temperatures were selected in this investigation, to investigate how much drug was recoverable after heating to different extremes. Heating to temperatures below the melting point of a drug is useful for drying purposes, and measurement of the melting point only needs the recording of the onset for this process. Only when the enthalpy of melting is required does the end temperature have to ensure completion of the transition. Degradation may, therefore, be minimised if the heating program is only required to melt the sample, as

demonstrated by the data given in figure 9 and table 2. The 30-minutes isothermal period applied in the TGA experiments not only allowed the prediction of stability but also mimicked manufacturing process conditions. For example, using hot melt-extrusion involves applying stress to materials with a rotating screw at temperatures above the melting temperature to achieve molecular level mixing of the active compounds and thermoplastic binders, polymers, or both (Patil et al., 2016).

The onset melting temperature of pilocarpine HCl exhibited a strong heating rate dependency, where the onset temperature started to increase with increasing heating rates. The observed melting point of pilocarpine HCl increased by 14 ^{o}C when the DSC heating rate was increased from 1 to 20 ^{o}C/min. Such dependence on the heating rate for reported melting points is not unusual. For example, melting point variation for sucrose, glucose and fructose with heating rate may be found in the literature; however, there are different causes for this dependency. Sucrose shows different onset temperature because of chemical degradation as reported by Lee (Lee et al., 2011a, 2011b), the process is complicated and strongly influenced by the DSC parameters selected, (Roos et al., 2013). The case is different with glucose and fructose as their heating rate dependency is due to significant superheating during melting rather than chemical degradation (Roos et al., 2013). The possibility of superheating occurring within the sample makes a strong case for the application of stage four, where the extent of any chemical degradation is measured.

The TGA-MS confirmed that salt disproportionation and subsequent loss of HCl gas is one of the significant reactions that occur when pilocarpine HCl's melts. Salt formation is a well-known approach to enhance the poor aqueous solubility of drugs (Nechipadappu and Trivedi, 2017). During the production of a solid dosage form, an API is usually subjected to harsh processing conditions such as wet granulation, drying, roller compaction, and compression (Thakral and Kelly, 2017). Due to these processing requirements, an API salt may disproportionate, i.e. dissociate and return to the less soluble, unionised form (Stephenson et al., 2011). Salt disproportionate has a negative impact on solid formulations, such as a reduction in the dissolution rate of the drug product (Mannava et al., 2016), reduction in the chemical stability of the API (Farag Badawy, 2001; Guo et al., 2000; Hsieh et al., 2014; Zannou et al., 2007), and alternation in the functionality of

excipients (Rohrs et al., 1999) which affects the physical integrity of solid dosage form (Box and Comer, 2008; Williams et al., 2004) Therefore, salt disproportion process must be identified at the early stages of the drug formulation to avoid the production of unsafe pharmaceutical products.

The Mohr's back titration method was used to investigate the presence of Cl^- in pilocarpine HCl subjected to heat stress conditions. The Ag^+ ions from $AgNO_3$ react with free Cl^- ions forming AgCl, and this reaction will proceed until the Cl^- is consumed, equation 1 & 2. Because the formed AgCl is more stable than the complex formed between Ag^+ and CrO_4 ions from the indicator, AgCl forms first as a result. With time as the titration progresses, all the free Cl^- ions form complexes with Ag^+ and any excess Ag^+ ion complexes the CrO_4 ions which result in the colour change (red participate) which represents the endpoint of the titration, equation 4 & 5.

$$Ag^+_{(aq)} + Cl^-_{(aq)} \rightarrow AgCl_{(s)} \quad (4)$$

$$2Ag^+_{(aq)} + 2CrO_4^{2-}{}_{(aq)} \rightarrow Ag_2CrO_4\,(s) \quad (5)$$

The visual judgement of the endpoint of titration contributed to a small window of recovery of Cl^- ions when compared with the expected and observed amounts for pure pilocarpine and pure NaCl. Despite that, the number of Cl^- recovered from the pre-heated pilocarpine HCl sample was lower than that of pure pilocarpine HCl. Titration confirms the hypothesis that HCl from pilocarpine HCl disproportionate under elevated temperatures, post melting.

The results from titration were complemented to the TGA-MS results. The MS detected the presence of Cl^- isotope 35 once the pilocarpine HCl nears and passes its melting point, figure 7. The raw DSC data, (supplementary 9.4), showed an endothermic baseline drift before the melting peak, which then overlays with the decomposition endotherm of pilocarpine samples. The endothermic bassline shift is mainly related to the loss of HCl salts from the heated pilocarpine HCl samples, as this shift was not noticed before the melting of indium and mannitol samples. Once above the onset of melting, pilocarpine HCl suffers significant salt disproportionation of produces HCl gas. The evolved HCl as it passes through the molten sample at high temperature must contribute to further chemical degradation as detected by HPLC.

Pilocarpine HCl was loaded into DSC pans prepared in two ways to investigate the effect of salt disproportionation on the relation between the onset melting temperature and the percentage drug recovery of pilocarpine HCl post-DSC cycle. The DSC pan was prepared as pin-holed non-crimped or pin-holed crimped DSC pans. In the case of the crimped pan, the hole was made to avoid the burst of the pan due to high pressure. The non-crimped pans were used for the ease of lid removal to aid in further chemical analysis such as HPLC. However, the crimped DSC pan is the standard method, and it is designed to prevent sample leakage pre and post DSC experiment (Gabbott 2008). The significant difference in the melting point between crimped and non-crimped is due to HCl salt disproportionation. Loading pilocarpine HCl in non-crimped DSC pans eases the release of HCl gas out of the pan. In the case of crimped DSC pans, the pin-hole limited the release of the HCl gas in comparison to the non-crimped. The disproportionation of HCl salts leads to volatilisation of HCl gas as reported in several studies (Elder et al., 2010; John et al., 2013). HCl gas either escapes from the "system" or reacts with the material that remains within the pan.

The trapped HCl will generate a higher concentration of degradation products, thus lowering the pilocarpine HCl recovery, see figure 8. A study conducted by Thakar 2017, tested the salt disproportionation in corticotropin-releasing hormone receptor 1 (CRH-1 HCl and CRH-1 HBr) using different heating rates by DSC (Thakral and Kelly, 2017). The study found that there is a reduction in the onset melting temperature of both samples with slower heating rates due to HCl and HBr salt disproportionation. This work supports that HCl salt disproportionation from pilocarpine contributes to melting point depression at slower heating rates (Thakral and Kelly, 2017). The results concluded from the application of stage #4 illustrated the importance of testing the chemical stability of the sample post DSC cycle for a valid melting point measurement.

Pilocarpine HCl is susceptible to isomerisation in aqueous solutions prepared for the development of ocular formulations. The initial reactions of this instability are the epimerization at the α- carbon of the lactone ring forming isopilocarpine. Hydrolysis of the α-lactone ring in pilocarpine and isopilocarpine produces pilocarpic acid and iso-pilocarpic acid, respectively (Gomez-Gomar et al., 1989), figure (11). As shown by Bundgaard & Hansen (1982), epimerization has only a small

contribution at room temperature in solution, but as the temperature increases, this contribution also increases (Bundgaard and Hansen, 1982). Thus, for pilocarpine HCl, it was pertinent to apply stage five to test the hypothesis that an as yet unreported epimerization could occur in the solid-state at an elevated temperature and in the liquid form as a consequence of melting. Very surprisingly both hypotheses were proved, heating both to 100°C and to 250°C under a range of heating rates resulted in a significant change in specific rotation indicating epimerization in the solid-state and the molten form, (table 2).

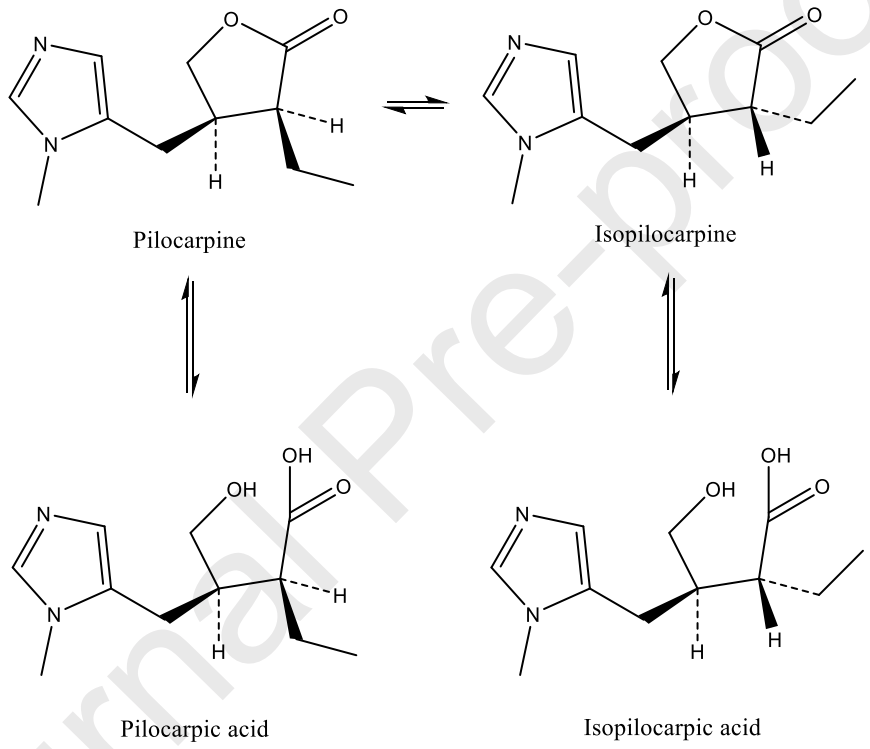


Figure 11: Degradation pathways of pilocarpine HCl in basic solution

The LC-MS peaks for the unheated and heated pilocarpine HCl samples both showed molecular ions or $[M+H]^+$ of 209.1, with a retention time of 1.8 minutes for the unheated pilocarpine HCl and 1.5 minutes for isopilocarpine HCl respectively. The molecular weight of pilocarpine HCl is 244.72 Da. However, when the pilocarpine HCl was injected in the LC-MS, the whole molecule was ionised and produced pilocarpine free base, yielding a molecular weight of 208.26 Da. The LC-MS modes used

were ESI – and ESI + represented as, $[M - H]^-$ and $[M + H]^+$, respectively. Therefore, the molecular weight of pilocarpine free base is recorded as 209.1 Da.

The appearance of a single peak related to pilocarpine or isopilocarpine or two peaks related to both of the materials in the LC-MS chromatograms is concentration-dependent. For example, the LC-MS chromatograms of the *in-vitro* prepared isopilocarpine showed a single peak, whereas the same sample showed two peaks in the HPLC chromatograms due to a low recovery percentage of pilocarpine, 3± 1.60. Therefore, the specific rotation was measured for better interpretation and support of the LC-MS results, (table 2).

The appearance of a new peak in the HPLC at retention time of 1.6 minute and the detection of two peaks in the LC-MS with a similar $[M+H]^+$ values of 209.1, along with the reduction of the specific rotation of the sample, provides strong evidence of a novel solid-state epimerisation for pilocarpine HCl. The extent of salt disproportionation can be evaluated by recording sample mass before and after exposing the API to high temperatures. For example, when pilocarpine HCl sample heated below their melting points using heat rate 10°C/min, the mass loss was very low, 2% ± 0.5 (w/w), thus salt disproportionation was low. When pilocarpine HCl was held just above its melting point, close to 215°C for 30minutes, only 53 ± 1.5% (w/w) of pilocarpine HCl was recovered from the pan, indicating the high salt disproportionation. Accordingly, the onset melting point ranging from 205°C to 206°C of pilocarpine HCl samples heated from 25°C to 250°C using heating rate 20 and 100°C/min represent the onset melting of the racemic mixture of pilocarpine HCl and isopilocarpine HCl, as the mass recovery was 99.91± 0.78% and 98.83± 0.94% for heating rate 20 and 100°C/min, respectively.

Pilocarpine HCl proved to be an excellent sample to explore the need for applying a multistage approach when measuring melting points. For pilocarpine HCl each stage was required, and their application has led to the discovery of a previously unknown solid-state epimerization. Although rare, solid-state epimerization does occasionally occur; for example, diastereomeric alpha nitriles will epimerize at 65°C while remaining in solid form (Sakurai et al., 2004). In the case of pilocarpine, the final melting point of a 205.5°C represents the onset of the transition of crystalline pilocarpine

HCl to a liquid racemic mixture of pilocarpine HCl and isopilocarpine HCl where disproportionation has been minimised because of the use of the relatively high, 20°C/min, heating rate.

6 Conclusion & Recommendations

Although DSC is commonly used to measure melting points in pharmaceutical science, the value of the observed melting point will vary if the drug is heat sensitive and the exact conditions used for the measurement of the melting point are not strictly controlled. The work reported here using pilocarpine as a case study has shown that the application of our five stage approach reduces this variance. In this study, pilocarpine HCl was characterised using PXRD, optical DSC, TGA, HPLC, DSC, back titration, TGA-MS, LC-MS and polarimetry. The visual observation of discolouration (stage1) was the first indication of the thermal instability of pilocarpine HCl. A significant mass loss through the melting region (stage2) indicated the loss of volatile products and was able to estimate the shelve life of the drug. The variation of the observed melting onset with heating rate (stage3) confirmed the heat sensitivity of pilocarpine HCl but also allowed extrapolation to a heating rate which minimised the impact of this instability on the observed melting point. In order to fully describe what the melting transition represents, and thus validate the measurement so it may be reproduced by others, the extent of the degradation (stage4) and potential for isomerization (stage5) must be characterised. The chemical analysis used to measure the extent of degradation indicated that pilocarpine HCl during melting suffers disproportionation, and the characterisation of the newly discovered solid-state epimerization showed that at the onset of melting the drug is nearly a racemic mixture of pilocarpine with its inactive form isopilocarpine. The conclusion shows that implementing the 5-stage approach is valid and can produce new stability data. Our 5-stage approach must be applied to all new drugs and for all formulation routes that demand heat, this is especially the case for most types of 3D printing, as thermal stability of the API is assumed firstly when extruded into a feed ribbon and then when melted within the printing head.

7 Acknowledgements

I want to thank Miss Sara M. Nasereddin and Mr Altin Kocinaj for their support with statistics.

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9 Supplementary data

9.1 Supplementary 1: Validation of Polarimeter instrument using Sucrose

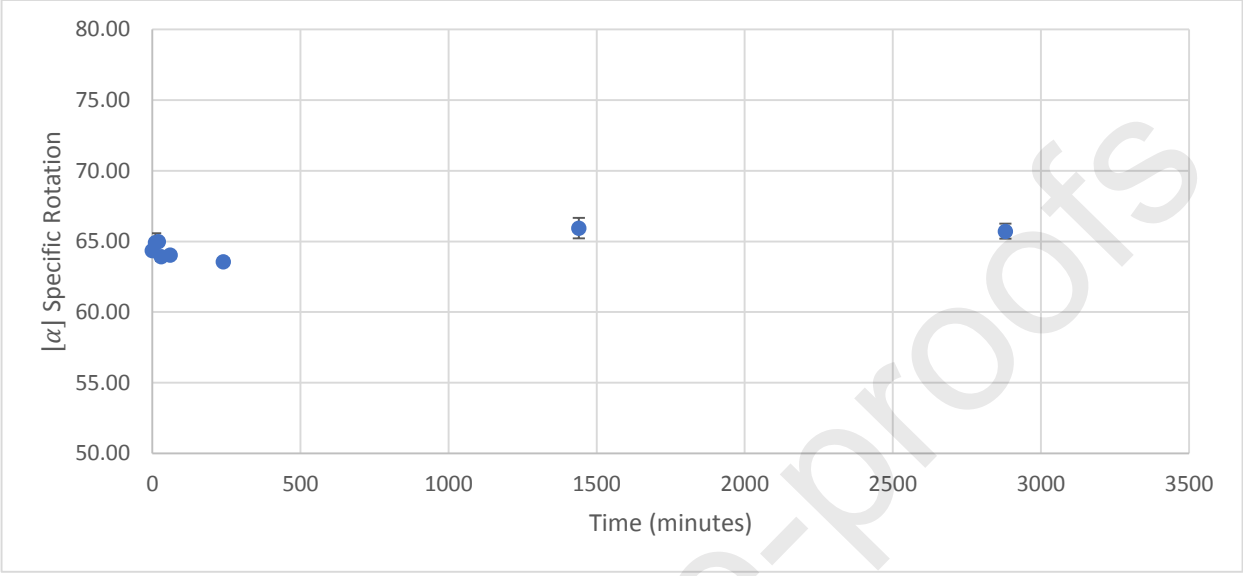

Figure S1A: Specific rotation of sucrose over time. A stock solution of sucrose was prepared by dissolving 26g of sucrose in 100 mL of HPLC water following the manufacturer's recommendation (ADP440⁺) n=3.

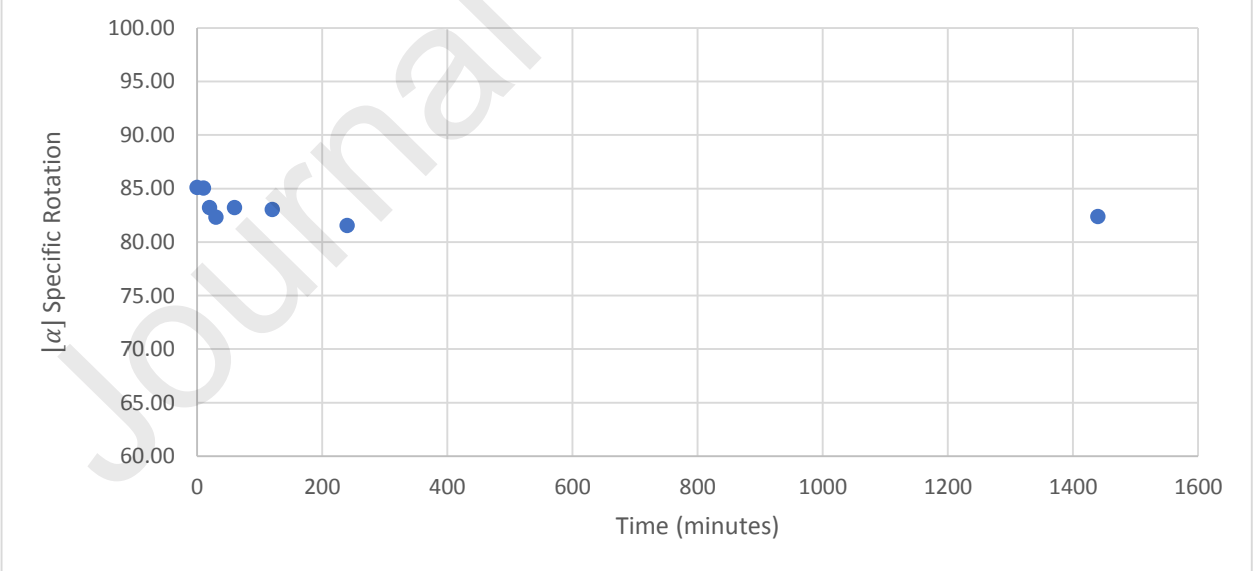

Figure S1B: Specific rotation of pilocarpine HCl in water over time. A stock solution of pilocarpine HCl was prepared by dissolving 0.2g of pilocarpine HCl in 20 mL of HPLC water, n=3.

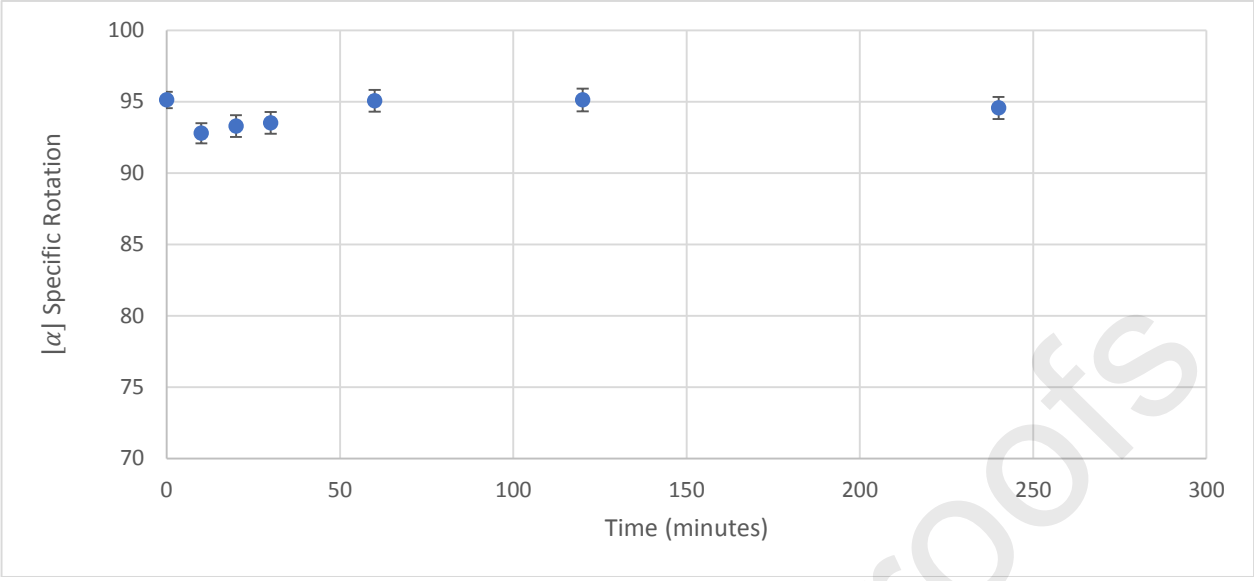

Figure S1C: Specific rotation of pilocarpine HCl in methanol over time. A stock solution of pilocarpine HCl was prepared by dissolving 0.2g of pilocarpine HCl in 20 mL of methanol HPLC grade, n=3.

9.2 Supplementary 2: PXRD of pilocarpine HCl

To determine the degree of crystallinity of the pilocarpine HCl powder, a diffractogram of the sample was recorded, with the diffraction patterns obtained being shown in Figure s2. The diffraction pattern shows the presence of peaks at the following diffraction angles, 2θ: 18.06°, 18.11°, 20.35°, 21.71°, 27.8°, indicating that the drug was present in the crystalline form, while the absence of diffraction peaks in the X-ray diffraction patterns of is indicative of the amorphous nature of any sample.

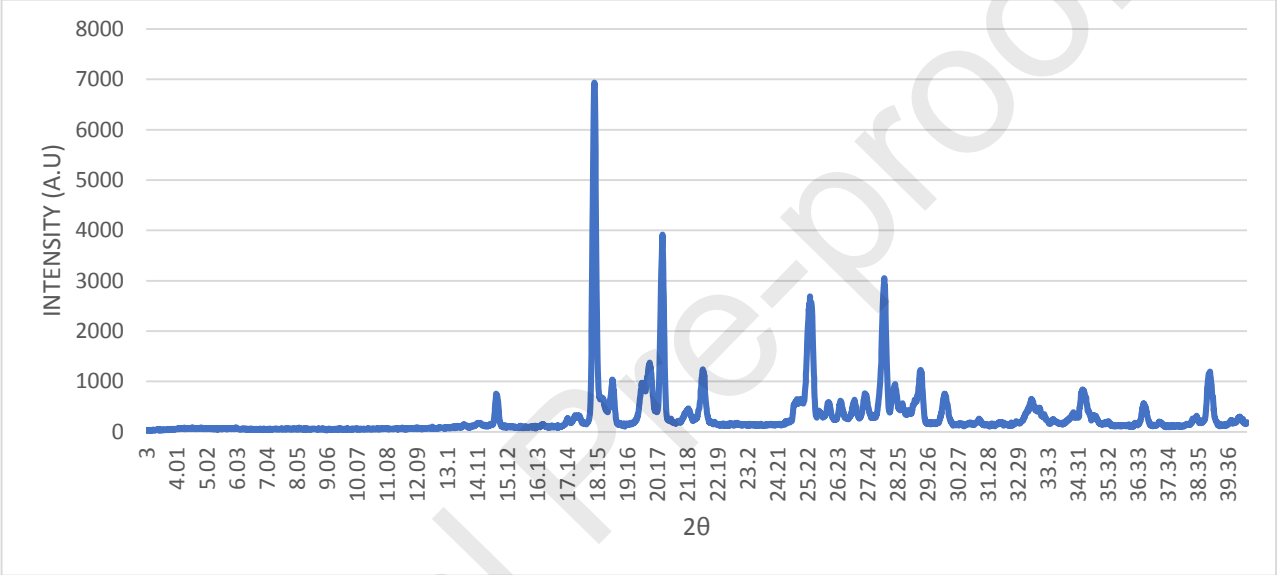

Figure S2: Powder X-ray diffraction of pure pilocarpine HCl powder.

9.3 Supplementary 3: Controlling DSC measurement

Table S3: Effect of different heating rates on the onset melting temperature of indium, mannitol and pilocarpine HCl. Samples weight was around 3mg ± 0.2mg. A number of repeats (n=3) for each material, ± represents the standard deviation between the sample repeat.

Heating Rates		1°C/min		5°C/min		10°C/min		20°C/min	
Sample Name	DSC pan	Onset temperature (°C)	Enthalpy (J/g)	Onset temperature (°C)	Enthalpy (J/g)	Onset temperature (°C)	Enthalpy (J/g)	Onset temperature (°C)	Enthalpy (J/g)
Indium	Pin-holed/crimped	156.72± 0.02	28.94± 2.68	156.54 ± 0.12	28.60± 0.94	156.68± 0.05	28.29 ± 0.55	156.70 ± 0.11	28.5± 2.54
Pilocarpine HCl	Pin-holed/crimped	192.05± 0.32	78.61± 31.96	200.56 ± 0.25	130.8± 4.72	202.66± 0.19	121.47 ±28.28	205.49± 0.34	128.7± 0.59
Mannitol	Pin-holed/crimped	165.94± 0.02	278.23 ± 22.66	166.00 ± 0.05	274.9± 6.28	165.75± 0.06	297.93 ± 2.39	166.00± 0.02	286.3± 3.56
Indium	Pin-holed/non-crimped	156.71± 0.02	28.78± 0.05	156.65 ± 0.01	27.90± 0.23	156.67± 0.04	28.04 ± 0.29	156.70± 0.01	29.35± 0.53
Pilocarpine HCl	Pin-holed/non-crimped	193.07± 0.05	143.27± 14.85	203.31 ± 0.09	134.0± 5.70	204.63± 0.06	148.23 ± 9.71	205.50± 0.35	141.8± 3.44

9.4 Supplementary 4: DSC raw data

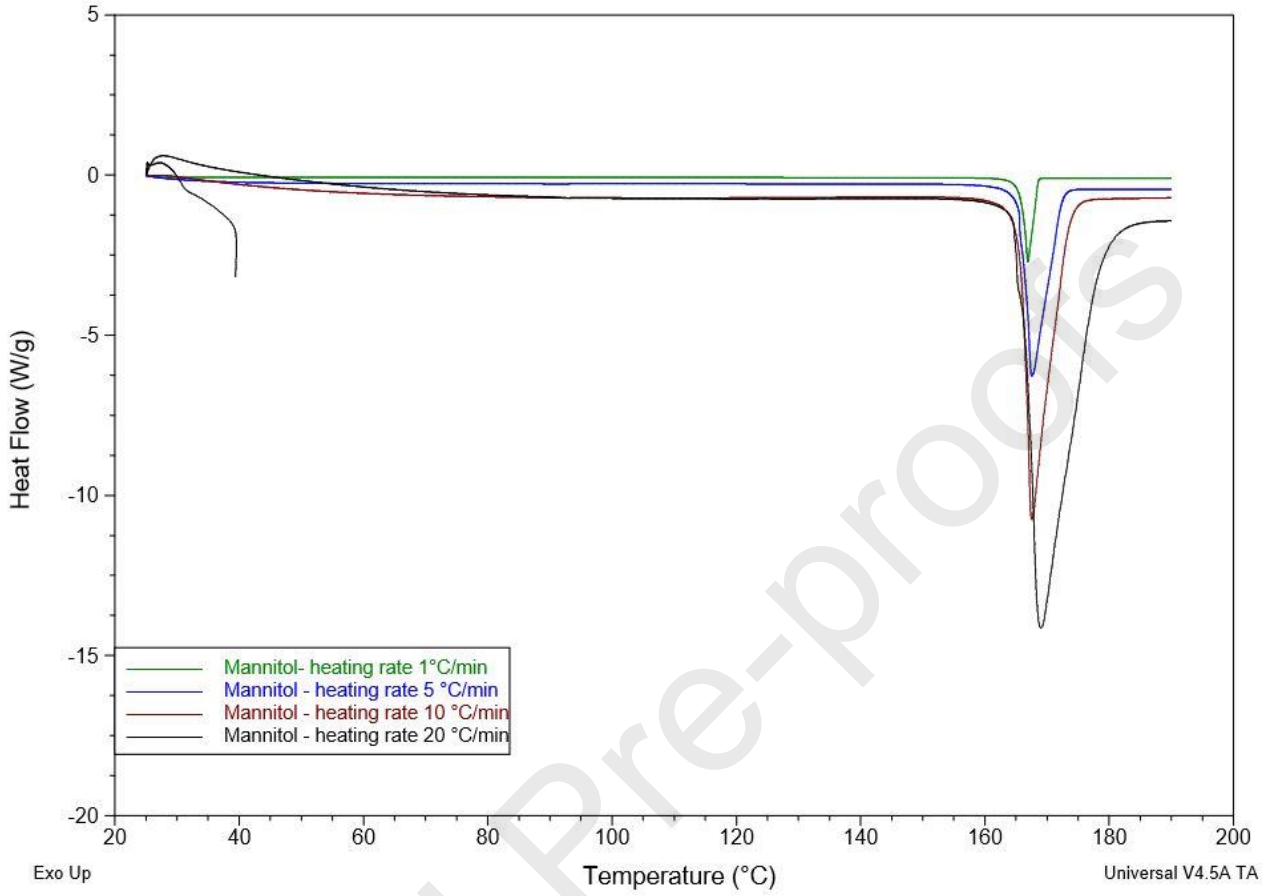

Figure S4A: DSC curves illustrate the effect of different heating rates on the melting point of mannitol. Samples weight was around 3mg, loaded into pin-holed crimped DSC pans, and heated from room temperature to 200℃. number of repeats was 3 for each heating rate.

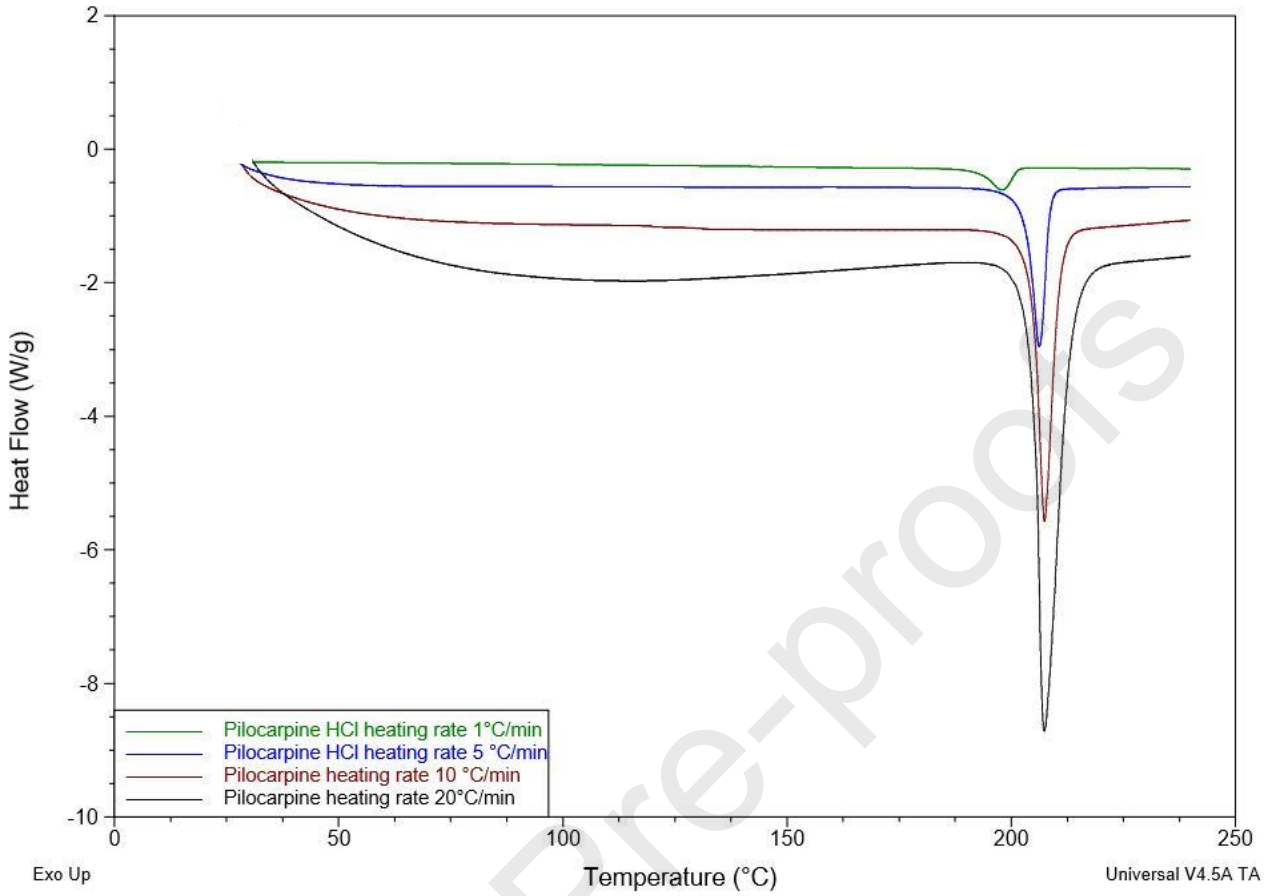

Figure S4B: DSC curves illustrate the effect of different heating rates on the melting point of pilocarpine HCl. Samples weight was around 3mg, loaded into pin-holed crimped DSC pans, and heated from room temperature to 250 ℃. number of repeats was 3 for each heating rate.

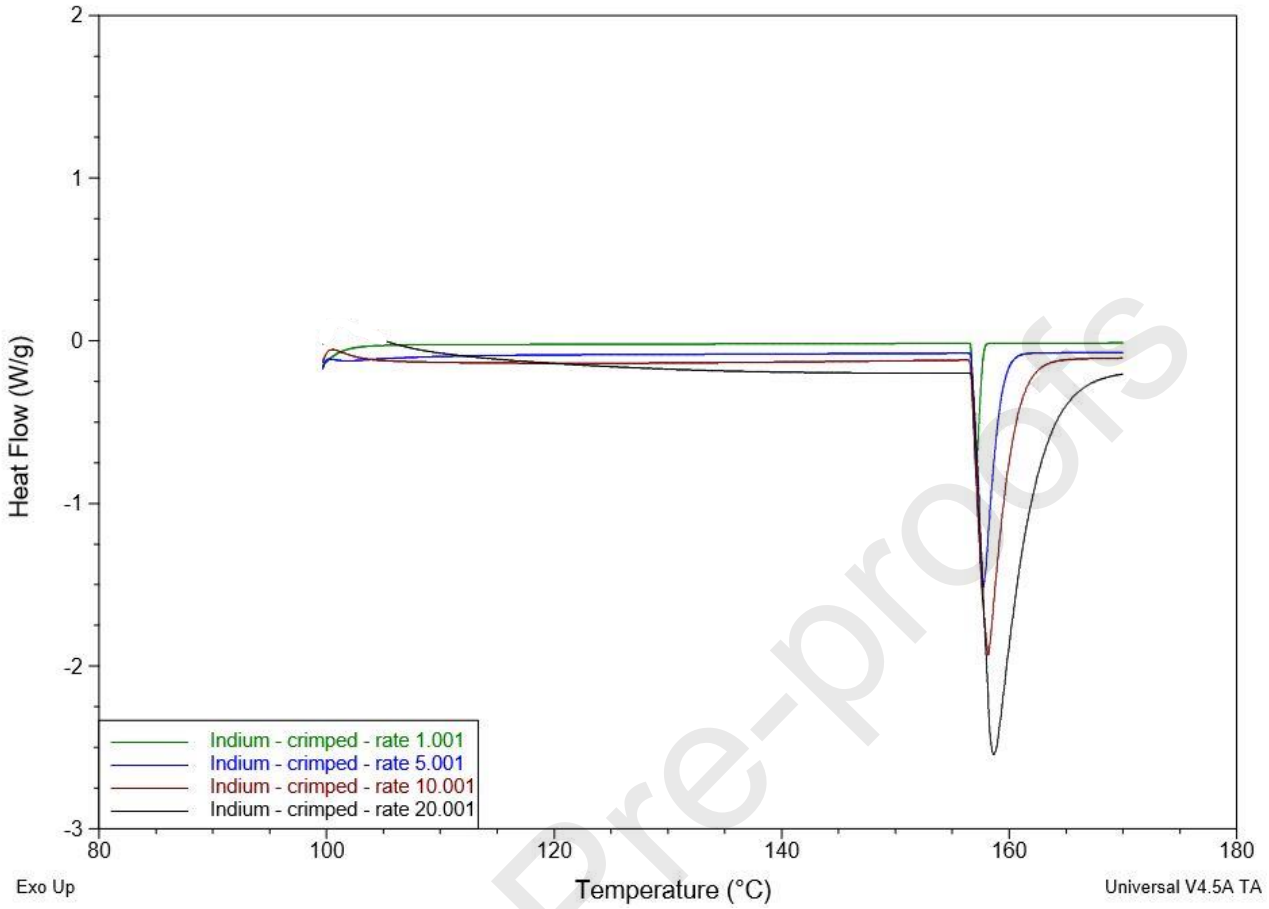

Figure S4C: DSC curves illustrate the effect of different heating rates on the melting point of indium. Samples weight was around 3mg, loaded into pin-holed crimped DSC pans, and heated from room temperature to 200℃. number of repeats was 3 for each heating rate.

9.5 Supplementary 5: Effect of different pilocarpine HCl weights on the onset melting temperature

Table S5: Effect of different pilocarpine HCl weights on the onset melting temperature and recovery percentage of pilocarpine HCl. All pilocarpine HCl samples were loaded into pin-holed crimped DSC pans and heated from 25°C to 250°C. A heating rate of 10°C/min was used. The results represent the mean value of n=3 for the onset temperature and enthalpy. Error bars determine from

Sample weight (mg)	Onset melting temperature (°C)
1	202.49± 0.09
5	202.34± 0.10
9	202.56± 0.20

9.6 Supplementary 6: The effect of varying target temperatures on onset melting and the recovery percentage of pilocarpine HCl

Table S6: The effect of varying target temperatures on onset melting and the recovery percentage of pilocarpine HCl. A number of tested samples (n=3) for each target temperature. All samples were heated using a heating rate of 10°C/min, with an isothermal period of 30 minutes once the end temperature was reached. Each sample was injected 6 times in the HPLC. The symbol ± represent the standard deviation between the samples repeats.

End temperature (°C)	Onset melting temperature (°C)	Recovery percentage
215	202.67 ± 0.13	53 ± 1.49
230	202.39 ± 0.73	16 ± 1.06
250	202.87 ± 0.21	± 0.52

9.7 Supplementary 7: Purity determination using DSC

The purity was measured using the DSC's built-in purity assessment tool to compare it with purity results generated by the HPLC. The measured purity for indium, mannitol and pilocarpine HCl using different heating rates are illustrated in figure s7. In general, all the three samples showed high purity post DSC run. The results showed that indium is very stable metal with very high purity, %100 with SD of % 0.004.

Regarding mannitol, the purity percentages start to increase with higher heating rates; however, these differences were not statistically significant. Finally, there was no relation between the heating rate and the purity percentage of pilocarpine HCl with a high standard deviation between samples repeats. The DSC purity assessment is suitable for metals and non-degrading organic materials in comparison with organic HCl salts, where the generated purity results were poor.

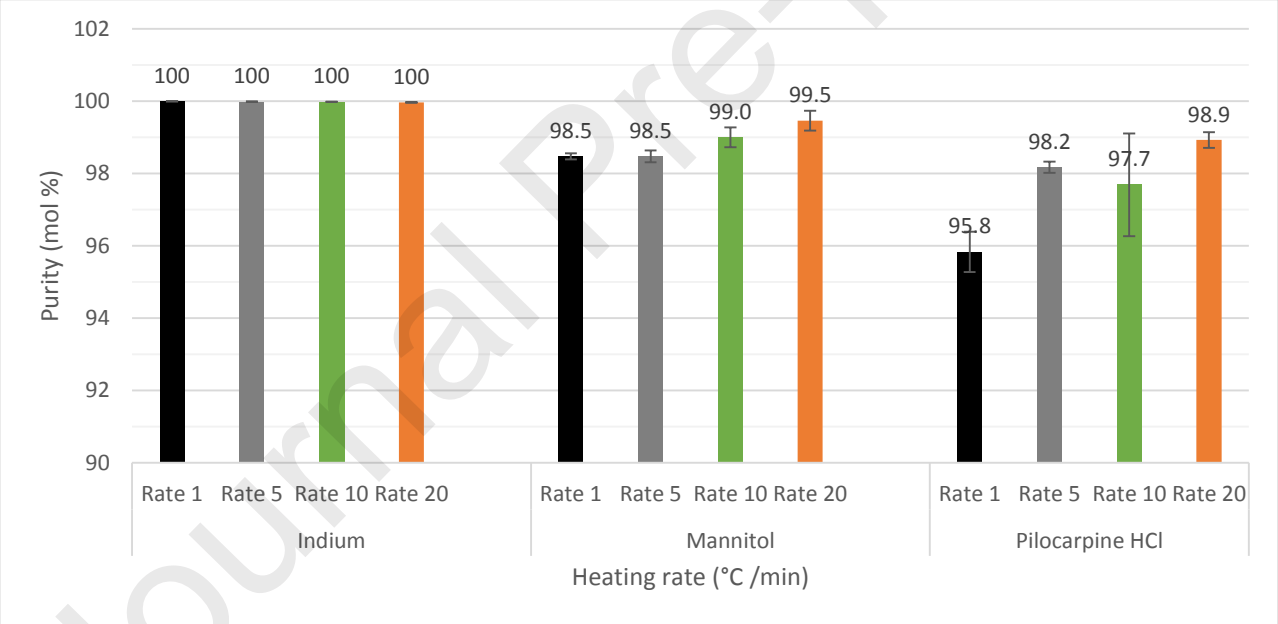

Figure S7: Post-DSC purity analysis of indium, mannitol and pilocarpine HCl using different heating rates. All samples weight was around 3mg, loaded into pin-holed crimped DSC pans, heated from 25℃ to 250℃ with isothermal for 30 minutes once the end temperature was reached. Error bard represents standard deviation between samples repeats, n=6 for each sample.