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The development of a quick method for amorphicity determination by isothermal microcalorimetry

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Abstract This study presents a contribution in the development of a quick and accurate testing method for the determination of amorphous content using isothermal microcalorimetry. Examples demonstrated how the choice of the experimental conditions, especially sample load, temperature and humidity, influences the crystallization of the amorphous material. The suitability of this systematic approach was first tested on well-known lactose and afterwards on nifedipine as model compounds. It was shown that by proper method design and careful selection of experimental conditions, it is possible to achieve quick determination of the amorphous content in samples with a quantification limit of less than 1%, what is considerably better than by classical analytical methods such as DSC and XRPD. Our optimized microcalorimetry method gave also better results compared to previously reported literature data for nifedipine.

Keywords Microcalorimetry · Amorphicity · Lactose · Nifedipine · Physical stability

Introduction

During the production of modern pharmaceutics, several technological processing of pharmaceutical solids are usually needed. These operations such as milling, mixing, spray drying, tablet compaction, and lyophilization can cause activation of the crystal structure, leading to various degrees of disorder [1, 2]. If this disorder is more extensive than the occasional molecular dislocation, it can be viewed as an amorphous region within the crystal structure. Because the amorphous state is in a higher energy state than the crystalline form, several physical changes can take place, resulting in several solid-state stability problems affecting drug product development, manufacture and shelf life [3].

Although the amorphous content, generated by the technological processing, can be less than 1% of the overall bulk, it is likely to occur at the powder surface, which is also one of the most critical and vulnerable regions available for sorption and other processes. In these cases, the amorphous regions in crystals can constitute reactive centres that can lead to product instability followed by changed bioavailability. Because of that, the ability to detect and quantify the amount of amorphous material within a highly crystalline drug is of great importance, concerning drug quality and performance.

Higher sensitivity of isothermal microcalorimetry (IMC), compared to analytical techniques, such as X-ray powder diffraction (XRPD) and differential scanning calorimetry (DSC), makes it a good and valuable alternative to the mentioned traditional approaches [4]. As in the case of DSC, IMC exploits the principle, that all physical and chemical processes are accompanied by the heat exchange with their surroundings. In spite of this similarity, there is a crucial difference between the two methods. In contrast to DSC, the sample in IMC is maintained under isothermal conditions within the microcalorimeter. The second difference is in the better sensitivity of microcalorimetry [5]. Both characteristics allow IMC to analyze a broader spectrum of sample masses under ambient storing conditions, without sample preparation, ageing or prestoring. If needed the analyzed sample can be further reutilized or retested using different techniques. Use of IMC therefore

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offers much potential in field of pharmaceutical science, not only in amorphicity determination but also to study other processes [6–9].

In this study, nifedipine and lactose were chosen as model materials as in both cases their amorphicity/crystallinity is of prime importance for their performance. Lactose monohydrate and nifedipine are found as fine and non-hygroscopic powder crystals while their amorphs exhibit completely different physical properties. The properties of amorphous material are namely highly influenced by the energy input changing the morphology and energy state of the material structure [10]. In our case lyophilization and quench-cooling were used for the production of amorphous lactose and amorphous nifedipine, respectively.

Lactose is a disaccharide composed of galactose and glucose. It exists in the form of two optical isomers, alpha- and beta-lactose. Today lactose powder is one of the most extensively used pharmaceutical excipient, usually used as a filler in the production of tablets and capsules. Because of this, its physical–chemical properties, such as hygroscopicity and powder flow, are of great importance.

Nifedipine [4-(2-nitrophenyl)-2,6-dimethyl-3,5-dicarbomethoxy-1,4-dihydropyridine] is a calcium channel blocking agent that is used to treat several cardiovascular disorders, such as hypertension and angina pectoris. Because of its low aqueous solubility, nifedipine shows low and irregular bioavailability after oral administration. Nifedipine has been shown to exist in three monotropic modifications [11]. Several attempts for increase of the oral bioavailability of nifedipine are possible, including the reduction of the drug's particle size, the development of solid dispersions with the use of organic solvents or by rapid cooling from the melt [12, 13]. In contrast to the poor water solubility of crystalline form of the drug, the amorphous nifedipine exhibits a higher equilibrium solubility and bioavailability, and it is often used as such.

In this study, IMC approach was examined for its ability to be used as a quick and sensitive method for the determination of amorphous content during physical stability testing. Lactose was used for the development of a suitable method which could be compared to previous literature data [14, 15] and nifedipine was chosen as its amorphicity/crystallinity properties are of prime importance for its performance and pharmacological use. The amorphous lactose and nifedipine were analyzed with the use of the miniature chamber technique. The influence of temperature, humidity and sample mass on the microcalorimetrical output was evaluated. Based on obtained results the optimal testing conditions were chose, enabling quick and sensitive quantification of the amorphous content. These testing conditions were further verified using a wide range of mixtures with known compositions.

Experimental

Materials

Amorphous lactose was prepared from the crystalline drug by lyophilization. Approximately 10 g of accurately weighed crystalline lactose monohydrate (Meggle) was dissolved in distilled water (10.0% w/v) by gentle heating and stirring for 10 min. The prepared solution was filled into a syringe and carefully dropped into a glass container filled with liquid nitrogen. The container was quickly transferred into a lyophilizator (Christ Beta 1–8 K lyophilizator) where it was left to dry for 24 h at 0.700 mbar and −10 °C. Before removing the sample from the apparatus, the cooling shelves were heated to ambient temperature. Until use, the samples were kept in a glass desiccator at room temperature.

Amorphous nifedipine was prepared from the crystalline drug (Sigma-Aldrich) by quench-cooling. The crystalline drug was melted on an aluminium pan by using an electrical heater. The melt was quickly purred into the liquid nitrogen and transferred into a desiccator connected to a water pump, where the drug reached the room temperature. The same desiccator was used for storing the samples until use.

Isothermal microcalorimetry

The calorimeter used in these studies was a MicroDSC III (Setaram) operated in the isothermal mode, with a standard Hastelloy 1 mL batch vessels which were modified into a miniature humidity chamber [14] (Fig. 1). A small, opened 100 µL glass tube was carefully placed into a standard vessel in the powder bed of the sample. Before closing the vessel, the glass tube was filled with 50 µL of water to obtain 100% relative humidity (RH) or a saturated salt

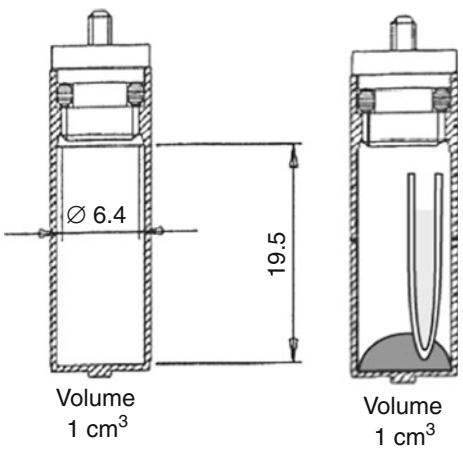

Fig. 1 Standard Hastelloy vessel (*left*) and miniature humidity chamber (*right*)

solution to obtain humidities below 100% RH. When the substances were tested in the absence of moisture, the glass tube was empty. The reference vessel was filled with an inert reference system (aluminium foil) equivalent to the sample mass, while the glass tubes in the reference and the sample vessel were filled with the same amount of solution.

In the investigation of amorphous lactose samples, the tests were performed at 25 °C, using 50 μL of distilled water in the glass tube. In the case of amorphous nifedipine samples, the tests were performed at temperatures 25, 35, 45 and 55 °C, using 50 μL of water, saturated KNO_3 or NaCl solution to generate different vessel humidities.

The heat flow (dQ/dt in μW) was measured as a function of time. By integrating the heat flow curve, the heat evolved or adsorbed (Q in mJ) was obtained. The calorimeter measured the heat conduction out of the sample cell to a heat sink so the output thermograms presented exothermic processes as positive heat flows and endothermic processes as negative heat flows.

The calorimeter was calibrated using the Joule effect method (as described in Micro DSC III User Manual by Setaram) in the range from 20 to 60 °C before experiment set.

XRPD

The samples were analyzed using a PANalytical X'Pert PRO difractometer with an alpha1 configuration (using a Jaohannson's monochromator) and Cu-Kα radiation. The samples were scanned in steps of 0.034° from 2° to 37°. XRPD was used to confirm the amorphicity of lactose and nifedipine. In the case of completely amorphous samples, no crystallization peaks can be detected.

Microscopy

Samples were examined before and after the microcalorimetric measurements by a stereo microscope (Olympus SZX12), under the 40,000 magnification. The stereo microscope was used to detect even small changes in the crystal morphology on the surface of the sample.

Results and discussion

Investigation of amorphous lactose and nifedipine samples

The amorphous lactose and nifedipine samples were investigated by XRPD. The results showed no crystallization peaks, confirming the suitability of the methods used for the production of amorphous lactose and nifedipine (Fig. 2).

A typical microcalorimetric trace for amorphous lactose is shown in Fig. 3, for which 35.0 mg of lyophilized lactose was loaded into a miniature humidity vessel, using 50 μL of water and tested at 25 °C. Because of the high humidity in the vessel, the data acquisition began soon after the vessel was closed and loaded into the measuring position; therefore some disturbances were present in the first part of the microcalorimetric trace.

From the investigation of amorphous lactose we could see that the microcalorimetrical traces are typically divided in three phases, as it can be seen from Fig. 3. According to literature data [14, 16], the observed phenomena can be interpreted as adsorption of water vapour in Phase I, crystallization in Phase II and release of the adsorbed water as a consequences of crystallization, characterized by Phase III [17]. Also in our case Phase I can be interpreted as adsorption of water vapour. Because the source of water vapour is housed within the sample vessel, the wetting of the powder does not exhibit a large response interfering with the crystallization. The reason for this is that vapourisation (of the housed water) reduces the exothermic signal produced by the water vapour condensing on the sample. Because the two processes are of similar magnitude, the result is seen only as a small composite peak that precedes the crystallization peak. Phase II was found to correspond to the actual crystallization process. The shape of this peak indicates a very rapid and cooperative process. The reason for this is that amorphous material is in a higher energy state and therefore adsorbs water vapour more easily than the crystalline parts. When the water content in the total amorphous structure has reached a concentration, at which the glass transition temperature has decreased below the experimental temperature, the amorphous parts cooperatively crystallize. This phenomenon clearly shown in thermogram (Fig. 3) is described also by several authors [16, 18]. The integration of the area under the crystallization peak, gave the equivalent to the total heat output for the process, which in the case of amorphous lactose was 42 J/g. The process involved in Phase III is still under debate. Some authors explain it as the release of water vapour from the powder sample as a consequence of the crystallization of the amorphous region [17], while others refer to as the mutarotation of β- to α-lactose with consequent crystallization [14].

After the IMC measurement, the morphology of the crystallized powder was investigated by the use of stereo microscope. The crystallized sample was found to be fused into one compressed lump of material, instead of round, free flowing spheres (Fig. 4). If the lump was gently ground, with a pestle in a mortar and reanalyzed under same IMC testing conditions, no response was observed. This confirmed that the crystallization of the amorphous material was complete.

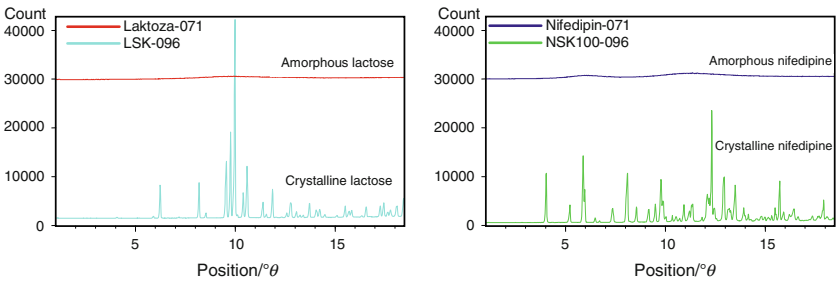

Fig. 2 XRPD scan of amorphous (*left*, *upper curve*) and crystalline lactose (*left*, *bottom curve*) and amorphous (*right*, *upper curve*) and crystalline nifedipine (*right*, *bottom curve*)

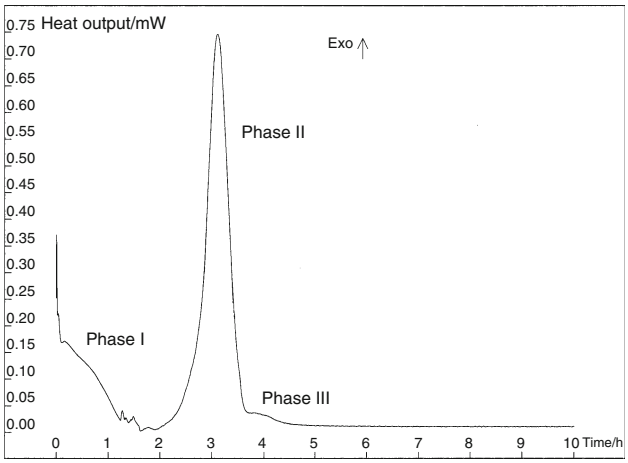

Fig. 3 Typical microcalorimetric trace of an amorphous lactose sample (25 °C, 100% RH)

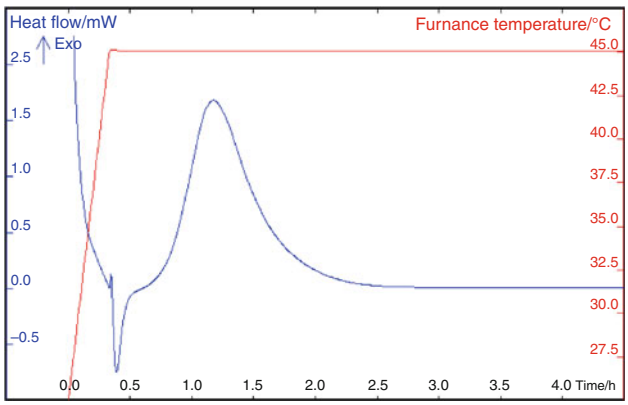

Fig. 5 Microcalorimetric trace of amorphous nifedipine sample (45 °C, 91% RH)

The same methodology but with different testing conditions, was applied in the investigation of amorphous nifedipine samples. Because of its poor aqueous solubility, the quench-cooling was used instead of the lyophilization to produce the amorphous nifedipine. A typical microcalorimetric trace for amorphous nifedipine is shown in Fig. 5, for which 64.7 mg of the amorphous drug was loaded into a miniature humidity chamber. Because the testing temperature was set to 45 °C, the heating phase (from ambient temperature) can also be seen. In the case of amorphous nifedipine samples, the microcalorimetric trace showed only two distinctive phases.

According to previous mentioned arguments in the case of amorphous lactose, Phase I (from 0.3 to 0.6 h) similarly represents the wetting of the sample powder, while Phase II (from 0.6 to 2.7 h) corresponds to the actual crystallization process (Fig. 5). In contrast to the microcalorimetric trace of lactose, the Phase III in the nifedipine trace is missing, showing that the crystallization process was even more cooperative. When comparing the Phase I for the two substances, another difference can be seen. As the Phase I, for amorphous lactose samples, approached the base line in a logarithmically transformed curve, the Phase I for nifedipine samples exhibits a distinctive endothermic peak before reaching the base line. The reason for this difference can probably be found in the different techniques used in the production of the amorphous substances. While lyophilization produces voluminous and bulky lactose, quench-cooling produces glassy nifedipine with much lower surface area (Fig. 6). Because of the larger specific surface of the lyophilized lactose, the vapour adsorption was quick and comparable to the vaporization process, in contrast to the slower adsorption by the glassy nifedipine sample. Consequently the endothermic heat of vapourisation prevailed the exothermic heat of condensation, resulting in a composite endothermic peak.

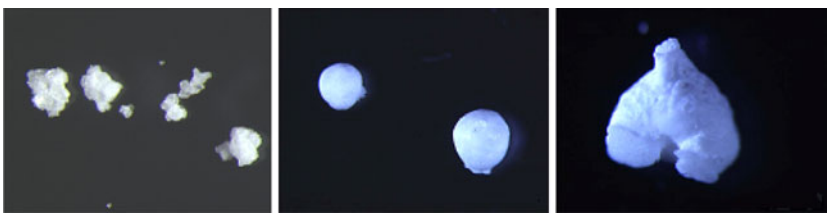

Fig. 4 Crystalline lactose (*left*), lyophilized amorphous lactose sample before (*centre*) and after (*right*) the microcalorimetric measurement

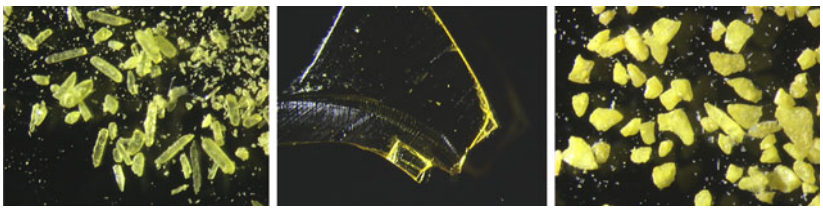

Fig. 6 Crystalline nifedipine (*left*), quench-cooled amorphous nifedipine sample before (*centre*) and after (*right*) the microcalorimetric measurement

In contrast to amorphous nifedipine, the crystalline and crystallized samples of nifedipine (Fig. 6) gave no response when reanalyzed under same IMC testing conditions. This confirmed that the crystallization of the amorphous material was complete.

On the basis of experimental results presented so far, we can confirm that IMC can be successfully used as a suitable method for the detection of the amorphous matter. In order to achieve sensitive and reliable quantitative results, it is very important to define and evaluate factors that influence IMC measurement, which was the subsequent aim of this study.

Factors affecting heat flow curve

The influence of sample load, which can be quite significant [14], was investigated by applying different loadings of amorphous lactose and nifedipine samples. Figure 7 represents the effects of different amorphous lactose loadings on the microcalorimetric outputs as a function of time. As it is seen from the figure, the increase of the sample load prolongs the time of crystallization, however, the enthalpy of the process (J/g) did not alter for different loads.

The same effect can be clearly seen in the case of amorphous nifedipine samples (Fig. 8), as well as the amount of sample increased, more time was needed for the sample to accumulate the required amount of heat to trigger crystallization.

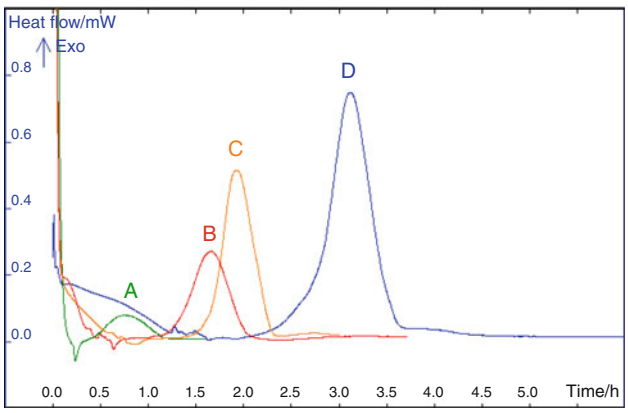

Fig. 7 Microcalorimetric traces for amorphous lactose samples (*A* 3.3 mg, *B* 10.5 mg, *C* 17.6 mg, *D* 35.0 mg)

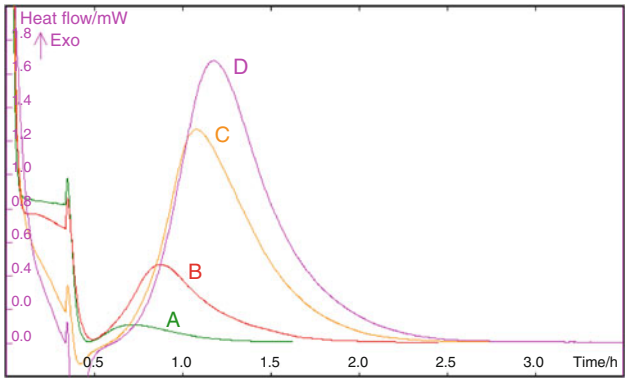

Fig. 8 Microcalorimetric traces for amorphous nifedipine samples (*A* 3.6 mg, *B* 15.4 mg, *C* 46.3 mg, *D* 64.7 mg)

As mentioned before, the crystallization of both substances was very cooperative. This means that crystallization does not take place until sufficient amount of water vapour is sorbed by the powder bed, lowering the glass transition temperature (T_g) and allowing the entire sample to crystallize. Larger sample loadings therefore need more time to adsorb sufficient moisture, what shifts the crystallization peak to the right and prolongs the experiment. In contrast to bigger sample loads, smaller loads or minor amorphous content can crystallize too soon, what can overlap Phase I and Phase II processes, impairing the sensitivity of the method. If overlapping occurs, experimental conditions (humidity and temperature) must be adjusted in order to delay the crystallization. Usually a decrease in the vessel humidity or the testing temperature is needed.

Although the effect of sample load is comparable for both amorphous substances, some difference can be seen. While the approximately fivefold increase in the lactose sample load (from 3.3 mg to 17.6 mg), increases the t_{max} nearly 2.5 times, the similar increase in sample mass (from 3.6 mg to 15.4 mg) prolongs the t_{max} less than 1.5 times for nifedipine. The reason for this phenomenon is probably the difference in the specific surface area of amorphous lactose and nifedipine. In contrast to the small specific surface area of amorphous nifedipine, the larger specific surface area of the bulky amorphous lactose needs more time to accumulate the sufficient amount of moisture what shifts t_{max} toward higher values.

The influence of temperature was also investigated, because it is known that temperature can significantly

affect the molecular mobility and therefore the crystallization process [10]. Raising the experiment temperature from 25 to 35, 45 or 55 °C drastically changed the microcalorimetric trace for nifedipine and shortening t_{max} from 540 to 180, 38 and 32 min, respectively.

According to our expectations, higher temperatures increased the molecular mobility and accelerated the crystallization of the amorphous matter. Quicker crystallization resulted also in higher and sharper crystallization peaks, increasing the experiment resolution. On the basis of our results, we suggest that in order to accelerate the experiment, the temperature can be raised, taking care not to change the mechanism of the studied process or affect the resolution. In case of nifedipine samples the temperature was set to 45 °C, what enabled quick and accurate testing of the amorphous matter, with a suitable separation.

Because even small amounts of adsorbed water can plasticize amorphous solids [10, 19], and therefore influence its solid-state properties, the effect of different relative humidities on the crystallization of amorphous nifedipine was also tested. Higher energy state of the amorphous material promotes sorption of considerably greater quantities of vapour than their respective crystals [14, 20].

The influence of humidity was investigated by measuring the amorphous nifedipine under various humidities and in the absence of an inner source of water vapour. Different humidity conditions were generated by filling a small glass tube with water to obtain 100% RH, or different saturated salt solutions to obtain humidities below 100% RH. In our case saturated NaCl and KNO_3 solutions were used to generate humidities around 75% and 91% RH, respectively [21]. In order to detect possible overlapping of the crystallization peak with Phase I, smaller samples were tested using an elevated temperature (45 °C). The influence of relative humidity was found to be inversely proportioned to the t_{max} of the crystallization peak, lowering the vessel humidity from 100% to 91%, 75% RH, prolonged t_{max} from 38 to 62, and 77 min, respectively. At 100% RH the crystallization occurred too soon, resulting in the overlapping of the crystallization peak and the Phase I, while no significant response was found within the first 10 h, when testing nifedipine without an inner source of water vapour. On the other hand, the 75% and 91% RH represented useful testing conditions for quantitative analysis of amorphous nifedipine.

The results indicate that the crystallization of amorphous nifedipine was enhanced by high temperatures and the presence of moisture in the cell, while the effect of the mass load was proportional to the onset of crystallization. In our case it was possible to lower the sample mass of amorphous matter even less than 1 mg, while still obtaining excellent separation and quantification properties. In this way, the influence of temperature and humidity, along with the loading mass were found to play an important role in the crystallization of amorphous nifedipine and can be therefore used as a valuable parameter in the optimization of the IMC testing method. The final experimental conditions for quantification of amorphous lactose and nifedipine were 25 °C/100% RH and 45 °C/91% RH, respectively. With these optimized testing conditions it was possible to get all the needed information at a lower temperature and in a much shorter time that it can be found in the available literature [22].

Quantification of amorphous content

The stated experimental conditions allowed quick and sensitive testing over a large scale of sample loadings varying from 1 to 100 mg, without affecting the resolution or mechanism of crystallization. In order to quantify the amount of amorphous content present in the sample, mixtures of known composition were prepared and analyzed. Figure 9 summarizes the results of microcalorimetric outputs for a batch of samples with known amorphous lactose content. In this case amorphous lactose was mixed with different amounts of crystalline lactose, the experimental estimate of the amorphous content was expressed as heat output (per sample mass; expressed as J/g) obtained during the crystallization process. The good determination coefficient ($R^2 = 0.9979$) and the intercept close to 0 of the regression line (Fig. 9), confirmed that the method was accurate and sensitive. The slope of 0.415 (expressed as J/g·%) corresponds to the specific heat of crystallization (41.5 J/g), which was obtained by testing the 100% amorphous samples and is comparable to literature data [16].

In the case of amorphous nifedipine loadings (from 1 to 97.6 mg) and mixtures with known amorphous content

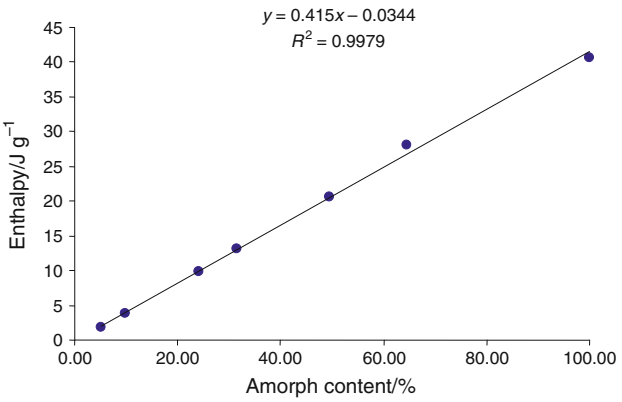

Fig. 9 Measured enthalpy of crystallization (J/g) as a function of amorphous lactose content

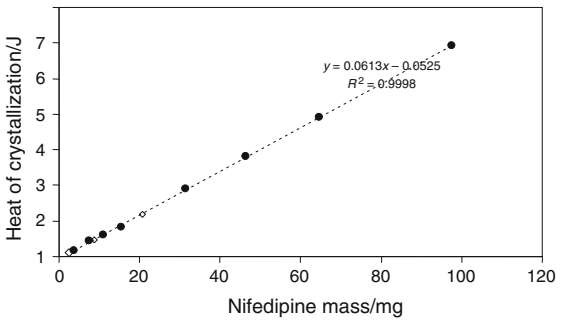

Fig. 10 Measured enthalpy of crystallization (*J*) as a function of amorphous nifedipine content. Tested amorphous nifedipine standards (*filled circle*) and mixtures (*open circle*)

were tested (Fig. 10). The obtained results excellently fitted the expected values, which were predicted from the trendline, constructed with the use of amorphous nifedipine standards. The determination coefficient for the varying masses of amorphous nifedipine was 0.9998, confirming a linear relationship between the known amorphous content and the measured enthalpy of crystallization. The intercept of the trendline was close to 0 and from the calculated slope the value of the specific heat of crystallization (61.3 J/g) was obtained. The method was further tested with the analysis of mixtures with known amorphous content where the total sample mass was 100 mg. All the tested mixtures matched the regression line including the mixture with only 1% of amorphous content (Fig. 10). These results confirm that the developed method is capable of detecting and quantifying even less than 1% of amorphous material in a sample.

With the mentioned statistical confirmation, the IMC method was found to be reliable, concerning linearity and sensitivity, and capable of quantifying amorphous content in unknown lactose and nifedipine samples.

Conclusions

The data presented in this study confirmed the role of microcalorimetry in studying amorphous and crystal properties of the solid state. It was shown that microcalorimetric experiments can be done isothermally at elevated temperatures or at ambient conditions, giving valuable complement to standard DSC information. With the use of different testing conditions, the influence of sample mass, temperature and humidity on T_g and crystallization was elucidated. The presented data emphasizes factors that are crucial for the optimization of a suitable IMC method. The optimal testing conditions, which allow quick and accurate quantification of amorphous content over a broad spectrum of sample loadings (from 1 to 100 mg), were found for both studied substances. The optimal conditions for amorphous lactose and amorphous nifedipine samples were 25 °C and 100% RH and 45 °C and 91% RH, respectively.

It was shown, that with proper experiment design and careful selection of the testing conditions, IMC could be used for the detection and quantification of even less than 1% of amorphous content. These performance characteristics and sensitivity can greatly improve the quality control activities in the production and selection of raw materials in dosage form design. All of this facilitates the optimization of pharmaceuticals and their stability as well, as the choice of the proper storing conditions, resulting in better product quality and longer shelf life. With such characteristics IMC represents a valuable and potent analytical method in the field of pharmaceutical development and quality control.

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