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AMORPHISATION METHODS AND INVESTIGATION OF THE AMORPHOUS STATE OF CLARITHROMYCIN

METODE AMORFIZACIJE IN PREUČEVANJE AMORFNEGA STANJA KLATIROMICINA

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I would especially like to thank my family and friends for the support they gave me during my studies. Cheers guys.

We live up to the potential we believe we have.

STATEMENT

I hereby state, that I have done this master thesis on my own under the mentorship of prof. Odon Planinšek PhD and Andrew Ingham PhD.

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ABSTRACT

The amorphous form of pharmaceutical materials represents the most energetic solid state of a material. It provides advantages in terms of dissolution rate and bioavailability. Literature reports several methods of solid-state amorphization (supercooling of liquids, milling, lyophilisiation, spray drying, dehydration of crystalline hydrates). Furthermore, there are several reports that amorphous forms of pharmaceuticals differ depending on method preparation and how these differences can be screened by a variety of spectroscopic (x-ray powder diffraction, solid state nuclear magnetic resonance, atomic pair wise distribution, infrared spectroscopy, terahertz spectroscopy) and calorimetry methods.

The aim of this study was to investigate whether chlaritromycin samples prepared by different methods (quench cooling, spray drying and cryo-milling) and different starting polymorphs of the drug (Form 1 and form 2) exhibit differences on the molecular level and how the percentage of the amorphous fraction can be evaluated when investigated by x-ray powder diffraction (XRPD), solid-state nuclear magnetic resonance (ss-NMR), differential scanning calorimetry (DSC) and attuned diffraction infrared spectroscopy (AT-IR). Both the quench-cooled and spray dried sample were fully XRPD amorphous, whilst on the other hand XRPD spectra of the cryo-milled samples still presented typical crystalline peaks until a sufficient milling time was met. Differences between the amorphous samples were seen using DSC. A significant difference between the quench cooled and the other two amorphous samples was seen in the AT-IR spectra, indicating a difference on the intermolecular level. ss-NMR was proven to be a suitable technique for the assessment of the amorphous fraction in samples, however the technique failed to detect any difference between the amorphous samples.

POVZETEK

Amorfna oblika trdnih snovi, ki se uporabljajo v farmacevtski stroki predstavlja energijsko najbolj bogato stanje trdne snovi. Sestavljena je iz neurejeno razporejenih molekul. Neurejenost in odsotnost kristalne rešetke imata za posledico, višjo entalpijo, entropijo in Gibbsovo prosto energijo amorfne oblike glede na kristalno. Značilnost amorfne oblike je temperatura steklastega prehoda. Gre za temperaturni interval, v katerem snov prehaja iz trdnega steklastega stanja v razmehčano stanje. V območju steklastega prehoda se poveča toplotna kapaciteta in mobilnost molekul, s tem pa je višja tudi verjetnost rekristalizacije. Višja entropija, entalpija in prosta energija vodijo do hitrejšega raztapljanja v primerjavi s kristalno obliko. Hitrejše raztapljanje je zanimivo predvsem za zdravilne učinkovine, katerih biološko uporabnost omejuje hitrost raztapljanja. Višja entropija pa ne vodi le k hitrejšemu raztapljanju, ampak predestavlja tudi oviro zaradi večje nestabilnosti in težnje k pretvorbi nazaj v termodinamično bolj ugodno kristalno stanje. Za amorfizacijo lahko uporabimo različne tehnološke postopke, katere lahko razdelimo v dve kategoriji glede na princip amorfizacije. V večini primerov snov najprej pretvorimo v termodinamični nestabilno nekristalno stanje (raztopino ali talino) iz katere v nadaljevanju pripravimo amorfno obliko s hitro ohladitvijo ali obarjanjem. Drugi princip je direktna pretvorba kristalne snovi v amorfno z razbitjem kristalne rešetke. Amorfno stanje iste snovi, pripravljene z različnimi metodami amorfizacije pa lahko izkazuje različne lasnosti glede na metodo amorfizacije. Te razlike lahko zaznamo z uporabo različnih tehnik, kot so rentgenska praškovna analiza (angl. X Ray Powder Diffraction -XRPD), jedrska magnetna resonanca v trdnem (angl. Solid state Nuclear Magnetic Resonance -ssNMR), infrardeča spektroskopija ter različnimi kalorimetrične metode.

Cilj magisterske naloge je bil najprej pripraviti amorfne vzorce klaitromicina z mletjem pri znižani temperaturi, sušenjem z razprševanjem in hitro ohladitvijo taline. Pri mletju lahko pride do prehoda zdravilne učinkovine iz ene polimorfne oblike v drugo, lahko pa tudi do delne ali popolne pretvorbe v amorfno obliko. Hkrati pa lahko pripravljena amorfna oblika prehaja nazaj v kristalno, zaradi lokalnega povišanja temperature pri mletju. Pri metodi hladnega mletja smo klaritromicin različno dolge intervale pri 4ºC s čimer smo preprečevali segrevanja in kristalizacijo učinkovine.

Kasneje smo te vzorce s ss-NMR vrednotili glede amorfnosti mletih vzorcev deleža. Druga metoda, katero smo uporabili za amorfizacijo klaritromicina je sušenje z razprševanjem. Metoda je primerna za pripravo amorfnih oblik, saj raztopino zdravilne učinkovine razpršimo skozi šobo v obliki drobnih kapljic v sušilno komoro, kjer pridejo kapljice v stik z vročim suhim zrakom. Razprševanje ustvari veliko specifično površino kapljic, ki ima za posledico zelo hitro izparevanje topila. Ker je izparevanje endotermen proces, se z upočasnjevanjem tega procesa zaradi nastanka trdne plasti zvišuje temperature kapljice, tlak v njeni notranjosti pa narašča. Hitro odparevanje topila onemogoči kristalizacijo molekul topljenca, ki posledično ostanejo v neurejenem amorfnem stanju. Hkrati pa izparevanje topila omogoči, da temperatura na površini kapljic ne doseže temperature steklastega prehoda in s tem pretvorbo amorfnega stanja v kristalino. Pri sušenju z razprševanjem, smo ustrezno količino klaritromicina najprej raztopili v etanolu, ki smo ga odparili v Buschi sušilniku z razprševanjem pri točno določenih pogojih. Amorfno obliko klaritromicina smo poskusili pridobiti tudi s hitro ohladitvijo taline. Amorfna oblika spojine nastane pri hitremu ohlajanju taline, v primeru, da je hitrost ohlajanja višja od hitrosti kristalizacije spojine. V našem primeru, smo v ta namenuporabili tekoči dušik. Pri tej metodi, je pomembno omeniti možnost kemijskegarazpada vzorca ob taljenju. Da bi se izognili temu razpadu in oksidaciji, smo taljenje klaritromicina izvajali pod dušikovo atmosfero. V nadaljevanju smo raziskovali ali se amorfni vzorci klaritromicina, pripravljeni z različnimi metodami amorfizacije (mletje pri nizkih temperaturah, sušenje z razprševanjem ter hitro ohladitvijo taline) in iz različnih začetnih oblik (Oblika I in II) razlikujejo na molekularnem nivoju. Razlike med amorfnimi vzorci klaritromicina smo poskusili detektirati z diferenčno dinamično kalorimetrijo (angl. Differential Scanning Calorimetry -DSC), infrardečo spektroskopijo-tehnika oslabljenega popolnega odboja svetlobe (angl. Attenuated Total Reflectance Infra Red -ATR-IR), ss-NMR, rentegnsko praškovno analizo. Poleg tega smo s ss-NMR poskusili kvantificirati amorfni delež klaritromicina v mletih vzoricih pri znižani temperaturi. XRPD in ss-NMR metode so pokazale, da sta vzorca pripravljena s hitro ohladitvijo taline ter sušenjem z razprševanjem popolnoma amorfna. To se je kazalo kot odsotnost značilnih XRPD vrhov v XRPD diffraktogramih ter razširitvijo vrhov značilnih za klaritromicin v ss-NMR spektrih. DSC metoda je zaznala steklast prehod manjšem obsegu pri vzorcih pripravljenih s

sušenjem z razprševanjem ter mletjem, izrazito je bil viden le pri vzorcu, ki je bil pripravljen s hitro ohladitvijo taline. Signifikantno razliko med obliko pridobljeno s hitro ohladitvijo taline ter drugima oblikama smo zaznali tudi z ATR-IR metodo. Rezultati nakazujejo, da se pripravljeni vzorci razlikujejo na intramolekularnem nivoju. V nadaljevanju, smo pokazali, da je ss-NMR tehnika primerna za kvantifikacijo amorfnega deleža v vzorcih klaritromicina mletih v različno dolge časovne intervale. Ta je nelinearno naraščal s časom mletja. Prav tako pa smo pri krajših intervalih mletja klaritromicina zaznali pretvorbo kristalne oblike I v bolj stabilno kristalno obliko II.

ABBERVATIONS

CM – Cryo-milled

CMC – Clarithromycin

C_p – Specific heat capacity

DSC – Differential scanning calorimetry

D_{bal} – Ballistic diffusion coefficient

D_{th} – Thermal diffusion coefficient

FT-IR – Fourier transform IR

H – Enthalpy

IGC – Inverse gas chromatography

MTDSC – Modulated temperature differential scanning calorimetry

MCC – Microcrystalline cellulose

NQR – Nuclear quadruple resonance

NIR – Near Infrared spectroscopy IR- Infrared spectroscopy

PDF – Atomic pair wise distribution

RAF – Rigid amorphous fraction

RS – Raman spectroscopy

SD – Spray dried

SEM – Scanning electron microscope

ss-NMR – Solid state nuclear magnetic resonance

T_{eff} – "effective" temperature

T_g – Glass transition temperature

T_c – Crystallisation temperature

T_m – Melt temperature

THF – Tetrahydrofuran

THz – Tetrahertz radiation waves

V – Volume

QC – Quench cooled

XRPD – X-ray powder diffraction

INTRODUCTION

The existence of drugs and excipients in multiple crystal forms (e.g., polymorphs) provides pharmaceutical scientists an opportunity to select the preferred form of the materials used in a formulation. This is very useful since critical properties, such as particle morphology and dissolution properties are frequently different between the different physical forms of a material. The amorphous form of pharmacologically active materials has received considerable attention because in theory it represents the most energetic solid state of a material, and should thus provide the biggest advantage in terms of dissolution rate and bioavailability (1). However, the amorphous state also presents various disadvantages such as a lower physical stability compared to crystals.

The structure of an amorphous solid is usually described as possessing crystal-like short-range molecular arrangement, but lacking long-range order. As illustrated by Fig. 1 the immediate environment of a molecule (*m*) in an amorphous solid may not significantly differ from that in a crystal (*e.g.*, similar number of and distance to the nearest neighbours), but an amorphous solid lacks any long-range transational-orientational symmetry that characterizes a crystal (1). However, this is only true when we are dealing with non-ionic material and substances that do not form strong intermolecular connections with water.

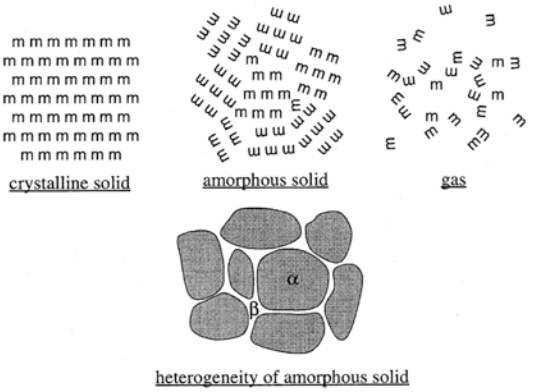

Fig. 1. Schematic representation of the structure of an amorphous and crystalline solid. The molecular arrangement in an amorphous solid is not totally random, as in the gas phase, but features short-range molecular order similar to that in a crystalline one. According to some models, an amorphous solid has distinct regions (*e.g.*, α and β), which have different densities and thermal relaxation behaviors (*i.e* the time scale for long-range molecular motion). Adapted from ref. (2) with permission.

In Fig. 2 the enthalpy (*H*) or specific volume (*V*) of a solid substance is plotted as a function of temperature. For a crystalline material at very low temperatures we see a small increase in enthalpy and volume with respect to temperature, which is indicative of a certain heat capacity (C_p) and thermal expansion coefficient. There is a discontinuity in both *H* and *V* at the melting temperature T_m representing the first order phase transition to the liquid state. Upon rapid cooling of the melt the values of *H* and *V* may follow the equilibrium line for the liquid beyond the melting temperature into a "super-cooled liquid" region. On cooling further a change in slope is usually seen at a characteristic temperature known as the glass transition temperature (T_g). At T_g the properties of the glassy material deviate from those of the equilibrium-cooled liquid to give a non-equilibrium state having an even higher H and V than the super-cooled liquid. The Kauzmann temperature (T_k) is considered to mark the lower limit of the experimental glass transition (T_g) (1).

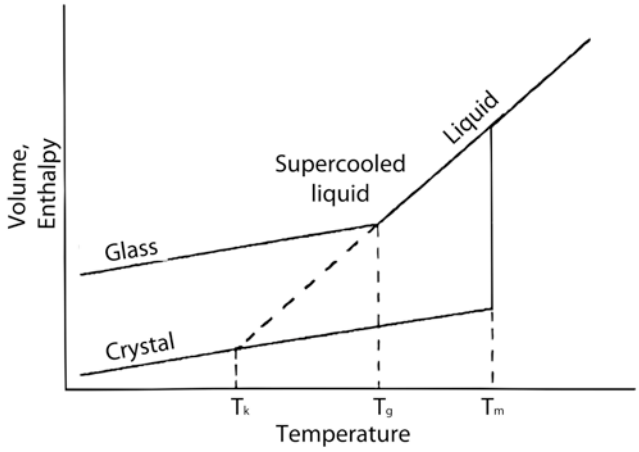

Fig. 2. Schematic description of the variation of enthalpy (or volume) with temperature. Adapted from ref. 1 with permission.

Amorphous materials have a higher Gibb's free energy than their crystalline counterparts, and as a result show a higher apparent solubility and faster dissolution rates, which in turn can lead to higher bioavailability for drugs that exhibit dissolution-rate limited absorption (classified according to Biopharmaceutical Classification System (BSC) as class II drugs) (3). However, due to the excess

entropy, enthalpy and free energy, that account for the better solubility, the amorphous state is inherently unstable and recrystallization may occur (2).

METHODS OF AMORPHISATION

The most common techniques to prepare amorphous form in pharmaceutical systems (*e.g.* pure drug or polymer glass solution) can be categorized according to two principal transformation mechanisms. In most cases the crystalline material is first intermediately transformed into a thermodynamically unstable non-crystalline form (either a melt or a solution), further on the thermodynamically unstable amorphous solid material is prepared by either quench cooling of the melt or rapid precipitation from solution *e.g.* spray drying. A second transformation mechanism involves direct solid conversion from the crystalline to the amorphous form. The best example for that is the mechanical activation such as milling. Whilst when melt and solution mediated methods are used all crystallinity is lost in the intermediate phase (melt, solution) mechanical activation methods may not cause complete disruption of the molecular order (1,4-6).

It should also be noted that differently prepared amorphous forms exhibit different properties. Salvonien *et al.* (7) have found that the cryo-milled simvastatin has a lower stability and decreased recrystallization rate than quench cooled samples of the same substance. In comparison, Karmwar *et al.* also found that the cryo-milled amorphous form of indomethacin is the least stable compared to amorphous forms prepared with quench cooling and spray drying (8). Moreover it should be noted the differences between the differently prepared amorphous forms are not only in the stability but can be detected on the molecular level using teraherz spectroscopy or solid state NMR (ss-NMR) (9,10).

The term polyamorphism has been used to describe amorphous states produced by different annealing times or preparative routes. An example are glasses that have been aged below T_g for different times and hence developed various degrees of “relaxation enthalpy”, that is the enthalpy of the aged amorphous substance (2). The term polyamorphizm has also been used in literature to describe the existence of distinct amorphous phases separated by first-order phase transitions (4,11). Patterson *et al.*

have shown that the susceptibility to amorphous conversion by different methods is compound specific (12). Therefore if amorphous conversion is required there is merit in investigating the use of more than one preparative technique.

Besides the possibility of the existence of polyamorphs for pharmaceutically relevant substances, the concept of the rigid amorphous fraction (RAF) has also been introduced. The RAF is believed to be an intermediate between the crystalline and amorphous phase. The striking difference between the RAF and the "true" amorphous fraction lies in the change of heat capacity (ΔC_p) at the glass transition (T_g). As RAF is more closely associated with the crystalline state, it does not undergo a change in C_p and the T_g. This implies that an amorphous form containing RAF will exhibit a smaller change in C_p and T_p than the same amorphous substance without RAF (13).

Solution based methods of amorphization

Melting and quench cooling. - Melting and quench cooling of a crystalline drug to produce an amorphous product is a relatively simple technique. Upon cooling of the molten drug below the freezing point molecular motion is slowed down. If cooling of the molten drug takes place fast enough, the molecules are not able to rearrange themselves into a crystalline lattice; therefore they are "frozen" in a more disordered state and crystallization is avoided (14,15). The slower a liquid is cooled, the longer is the time available for configurational sampling at each temperature, and hence the colder it can become before falling out of liquid (7). Therefore the enthalpy of the end product depends on the cooling rate (16), although it must be noted that if the cooling rate is too slow, the product will crystalize. The drawback of this approach is its potential for chemical degradation during the melting step due to high temperatures, as the degradation products may lower the glass transition temperature of the amorphous product (17). Thermal degradation is compound dependent and only limited steps can be taken to overcome this problem such as heating under an inert gas (12). So far a range of pharmaceutical active substances and excipients have been successfully amorphised by melting and quenching (18-21).

Spray drying. - The spray drying technique may be suited to obtain amorphous form of the drug substance, either alone or in combination with polymer (22-25). It

converts a liquid solution into a powder in one step (26). A concentrated liquid is pumped to the atomizing device where it is broken into small droplets. These droplets meet a stream of hot air and lose the solvent very rapidly while still suspended in the dry air. Components of the concentrated liquid may not crystallize immediately when their solubility limit is reached at the surface of the droplet. In this case a fully or partially amorphous solid is formed (27). Drugs that have a relatively low T_g, make it very difficult to obtain stable amorphous product in the form of a free flowing powder by spray drying. As the outlet temperature rises above the T_g, there is always a possibility that the final product is present in the super cooled rubbery state. Also such product is often sticky or tacky, which causes a decrease in product recovery and hampers handling in subsequent processes (22). Spray drying has commonly been used in the past decade as a technique to prepare amorphous active substances and excipients (28-31).

Freeze-drying. - Freeze-drying, also known as lyophilization, has been used as a pharmaceutical unit operation for a number of years for the low temperature drying of injectable systems (32). Freeze drying involves the desiccation of a substance by crystallization of water, followed by sublimation of water vapour from the solid state at reduced pressure. Depending on the cooling rate some solutes may crystallize during the freezing stage. Those solutes that do not crystallize are converted to amorphous solids when the temperature drops below the T_g of the maximally concentrated solute. At the end of a freeze-drying process, when the solvent is completely removed through sublimation, the freeze-dried formulation exists as an amorphous or partially amorphous system. The T_g of a freeze-dried formulation is determined by components of the formulation and the presence of residual water, which can act as a plasticizer lowering the glass transition temperature (33). Recently the process has been utilized to produce amorphous trehalose, which is commonly as an excipient in the field of lyophilisation and itraconazole (34,35).

Solid state amorphization

Dehydration of crystalline hydrates. - Dehydration of crystalline hydrates has been demonstrated as a feasible and “gentle” route to the amorphous state of organic solids. Saleki-Gerhardt *et al.* showed that heating the crystalline raffinose pentahydrate at 60

ºC in vacuum converts the material to an amorphous form identical to one produced by freeze drying (5). Li *et al.* observed that the crystalline carbamazepine becomes amorphous upon dehydration at 45 ºC with N_2 purge (36). The resulting amorphous solid undergoes a glass transition at 56ºC, which is markedly above the drying temperature (45 ºC), and crystallizes on further heating (at 86 ºC). These studies indicate that apart from being a potential route to amorphous solids, the drying of crystalline hydrates may reduce their physiochemical stability through the loss of crystallinity. More recently Sussich *et al.* investigated the amorphisation of a di-hydrate crystalline polymorph of trehalose upon dehydration (37).

Milling. -Milling, also known as comminution or grinding, is typically regarded as a particle size reduction process across many industries, including manufacturing pharmaceuticals and fine chemicals (38,39). In secondary pharmaceutical processing, it is often used to increase the specific surface area of poorly water-soluble drugs, so as to improve their dissolution properties and bioavailability (40). There is a variety of different commercially available milling equipment that can be utilized for the communition of pharmaceuticals. Generally the devices can be subdivided into three main categories, based on how energy is transferred to the material to be ground: ball mills, shear action mills and shock action mills. In the case of ball mills, energy is transferred to the ground material by mill bodies or impellers. The material is exposed to both shear and normal stresses. On the other hand in shear action mills, the material is ground by crushing elements (solid surfaces in relative motion). Shock action mills transfer the energy directly to the milled material. In this case material is milled by direct collision of particles. However, besides reducing the particle size, the milling process is often accompanied by other unintended effects such as changes in morphology (41), crystallinity (41), polymorphism (42), glass transition temperatures (43), chemical stability (44) and melting behavior (45) during subsequent post-milling storage. One must also consider that the process of milling is very energy-consuming. Furthermore, it would also be possible that the high energy output could result in degradation of the milled substance and the milling equipment. Milling is also used for more specific applications such as preparation of co-crystals (46,47). Pharmaceutical literature describes numerous examples of organic compounds like piroxicam (48), budesonide (49), sucrose(50,51), lactose (52,53), trehalose (54,55),

which become partially or completely amorphous when they are submitted to milling. However, it appears that one of the disadvantages of amorphisation trough milling is that the T_g of the milled substance plays a fundamental role and sufficiently low temperatures of milling (T_{mill}) must therefore be met to induce amorphisation through this process (56).

Principles of solid-state amorphization trough milling. - There are several competing ideas as to how the transformation from the crystalline to the amorphous state takes place during milling. A commonly held view is that amorphization occurs via the generation of localized heating effects followed by quenching. Alternatively Okamoto *et al.* have suggested that milling leads to an increase in static disorder that adds to the intrinsic dynamic disorder inherent within the lattice up to a critical value where the structural collapse occurs (57). Others argue that the disordering process is best regarded as an accumulation of defects (or, viewed another way, a dramatic reduction in crystallite size) and hence may not necessarily be regarded as amorphization in the traditional sense (58). Some of the advantages of cryogenic milling are that it is a mild method of producing amorphous material without the use of solvents or melting.

Influence of temperature on solid-state amorphization through milling. - The group of Descamps *et al.* provides a very significant contribution on how previously mentioned theories apply to organic crystalline materials (52,59,60). They have highlighted the relationship between the temperature of milling and the glass transition temperature of the corresponding material. In particular, they have demonstrated that cryo-milling (well below the T_g) results in amorphization and milling above T_g results in polymorphic transformation. This contradicts the melt-quenching explanation. These authors favour the concept of driven materials, as outlined by Martin and Bellon (61). In brief, the approach suggests that on milling a material there is a temperature independent disordering process induced by ballistic interactions, which competes with temperature dependent restoration process. The balance of these two is expressed in terms of an “effective” temperature T_{eff} which is the temperature at which the non-milled material would assume the same level of disorder as the milled one. A milling process that resulted in a T_{eff} above the melting point would therefore be expected to result in amorphization. The value of T_{eff} may be estimated via *Eq.* (*1*) where T is the temperature of milling, D_{bal} is the (temperature independent) ballistic diffusion

coefficient and D_{th} is the thermal diffusion coefficient corresponding to the restorative process.

$$T_{eff} = T(1 + \frac{D_{ball}}{D_{th}}) \quad (1)$$

It is significant that this theory predicts that at low temperatures D_{th} becomes small compared to D_{ball} hence raising T_{eff}. This then explains the counterintuitive observation that amorphization occurs to a greater extent at lower temperatures (62).

A related question is whether the material generated by cryo-milling behaves in a similar manner to "conventionally" generated amorphous materials. Surprisingly little is known on the subject although a thorough study by Crowley and Zografi, indicated that cryogenic milling of crystalline indomethacin resulted in amorphous material with similar T_g values but reduced physical stability compared to quench cooled amorphous indomethacin. The authors ascribing the latter to the presence of residual crystalline material(63). Qi *et al.* have also demonstrated the very marked instability of the cryo-milled material below its T_g and also the possibility of the prepared amorphous materials exhibiting a more complex recrystallization profile that is commonly considered for the solid state amorphous form (62).

The effect of crystal morphology on induced crystal disorder trough milling. - One persistent challenge in the development of pharmaceutics is the crystal habit, or morphology. For example equidimensional crystals are usually preferred in the industry as they have better handling and processing characteristics, such as flowability and compactability (64,65). Chikalia *et al.* have found that ß-succinic acid in a plate like morphology is more prone to disorder than a needle like morphology (66). Crystal morphology engineering and the use of crystals with the most suited morphology could therefore be a valuable tool to enhance the solid-state transformation through milling.

Co-milling. - Co-milling of drugs with excipients has been employed for acceleration of solid amorphization and stabilization of amorphous state. Watanabe *et al.* showed that amorphization of indomethacin could be achieved by milling it with polyvinylpyrolidone or silica (67). Ali *et al.*, used a vibration mill to prepare amorphous co-ground mixtures of flufenamic acid with amorphous calcium silicate

and silicon-dioxide (68). Amorphization of ibuprofen, sulfathiazole, phenothiazine, acridine, chloranil and vitamin K3 has been achieved by co-grinding with polyvinylpyrrolidone (69-71). Amobarbital amoprhised in the presence of variety of excipients such as carbon black, ethyl cellulose, precipitated silica and activated charcoal (72). A variety of other excipients such as ß-cyclodextrins, dextrans, chitin, chitosan, gelatin, polyethylene glycol, methyl cellulose, hydroxyl propyl cellulose, calcium silicate and silicon dioxide were used to amorphize structurally diverse drugs resulting in various degrees of amorphization (67-77). More recently Bahl *et al.* have shown that increasing the amount of Neusilin US2 with respect to indomethacin reduced the amorphization time (Fig 3). The mixture also showed a high physical stability (78).

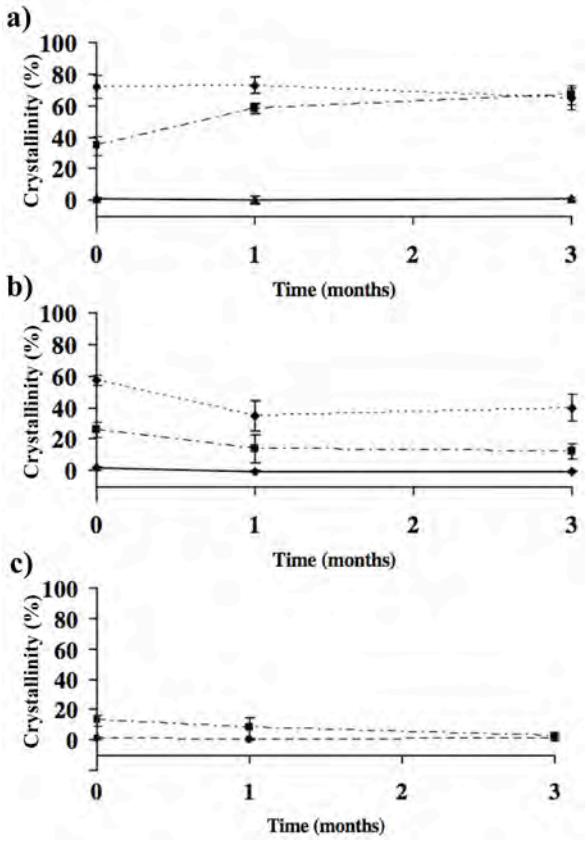

Fig. 3. Indomethacin co-ground with Neusilin in different ratios at 75% RH and stored 40ºC/75% RH: (a) 1:1 (b) 1:4 (c) 1:5 Key: 12 h co-ground - - - - - - 3 days co-ground - -- -- -- 5 days co-ground 8 days co-ground. Adapted from ref. 78 with permission.

Time of milling and solid state amorphization. - The conversion of crystal to glass upon milling often requires milling times of several hours to complete (50,54). Short milling times are thus expected to induce a size reduction of both the crystallites

(small single crystals) and the particles (a particle can be composed of several crystallites) without generating a noticeable amorphous content. Until now, only a few investigations of weakly milled materials have been performed and little is known about the structural and micro structural states that precede the amorphization observed during long and intense milling (79). A very important point is to determine when and how the accumulation of crystalline defects (crystal surfaces, dislocations, vacancies etc.) upon milling gives rise to a genuine amorphous state, which is, on the contrary, characterized by chemical disorder. Caron *et al.* have studied the structural and thermodynamic changes of crystalline alpha-lactose in the course of its solid-state amorphization by milling. The results revealed that, in the course of the milling process, the material cannot be described as a biphasic system made of both perfect crystalline matter and genuine amorphous matter. It appears, to be constituted of a wide panel of structural states more or less disordered and ranging from the crystalline state to the amorphous state. This conceptual difficulty emerges clearly from the results of these authors who have shown that very different characterization techniques give rise to very different kinetics of amorphization (Table I) (10,80,81). In a study Terada *et al.* have shown that the crystalline form milled had an influence on time needed for amorphisation trough milling, as that the peak intensities of the X-ray powder diffraction (XRPD) patterns of two different polymorphs of terfenadine decreased with increasing grinding times (82) (Fig. 4).

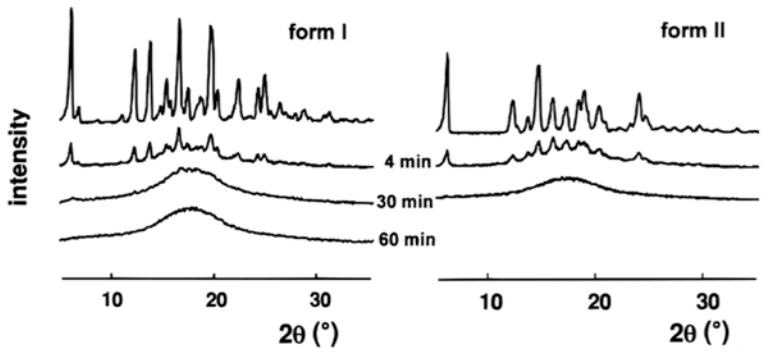

Fig. 4. Change in XRPD patterns of terfenadine (form I and form II) by grinding. Adapted from ref. 82 with permission.

Table I Differences in time of amorphisation depending on method of solid-state characterization.

	Indomethacin	Lactose	Trehalose
DSC	120min	50h	10h
XRPD	30min	1h	No data
ss-NMR	195min	30h	20h

CHARACTERISATION OF THE AMORPHOUS STATE

The strategy of characterizing amorphous solids differs from that for crystalline solids. Molecular-level structural elucidation, as is feasible for crystalline solids by diffraction and spectroscopic methods, is less applicable to amorphous solids, and greater emphasis is placed on structural mobility and changes. It is customary that amorphous material is characterized both below and above the glass temperature *i.e.*, both as the frozen solid and the super-cooled viscous liquid. Thus the effective characterization of amorphous pharmaceutical products requires a multidisciplinary approach using complementary analytical methods. To characterize pharmaceutical solids we may use various techniques, such as Raman spectroscopy (RS), Fourier transform infrared spectroscopy (FT-IR) or solid state nuclear magnetic resonance (ss-NMR), which are primarily intramolecular methods probing the sample at the molecular level. Intermolecular information is gained by directly employing techniques such as differential scanning calorimetry (DSC), thermogravimetric analysis (TGA), and XRPD, which analyze the sample on a particulate level. Recently tetraherz pulsed spectroscopy (TPS), second harmonic generation (SHG) and ^{14}N nuclear quadrupole resonance (NQR) have also been used as spectroscopy techniques to directly investigate particulate properties of solids (81,83-85). Other properties associated with the particulate level such as morphology or size distribution can be characterized using microscopic techniques such as polarizing light microscopy (PLM) and scanning electron microscopy (SEM) (84,86).

These methods offer several types of information about the investigated substance (87,88):

(i) Structure: The structure of amorphous solids is not random at the molecular level, but may present short-range order, residual crystallinity, polymorphic states, and regions of different density.

(ii) Thermodynamics: As mentioned before amorphous solids present higher energy, entropy and free energy when compared to the crystalline state of the same material.

(iii) Changes: In the right conditions amorphous solids can crystallize or undergo structural relaxation owning to the instability with respect to the corresponding crystals and "equilibrium" glasses.

(iv) Multi-component systems: Many pharmaceutical formulations are formed by either active substances and drug excipients or multiple active substances. One or more of the components can be present in the amorphous state.

Solid-state nuclear magnetic resonance

Recently ss-NMR has been introduced to identify effects such as polymorphism, intra and inter-molecular hydrogen bonding and tautomerism and is now widely used in conjunction with other analytical techniques (89). High-resolution ^{13}C ss-NMR spectra are obtained using proton decoupling and magic angle spinning (MAS) and sensitivity enhancement is achieved by cross-polarization (CP). ^{13}C ss-NMR has the advantage of being a nondestructive test method that provides information about the structure of the material (90). Furthermore, one of the advantages of ss-NMR is that it is very sensitive to minor conformational changes but is insensitive to particle size. In a ss-NMR study of ranitidine hydrochloride, the authors showed that form II of the drug exhibits molecular disorder in crystals and contains two tautomers, nitronic acid and enamine (91). The molecular disorder was attributed to ranitidine hydrochloride solved intermolecular bonding (89). For the charactersiation of organic substances, the remarkable sensitivity of the ^{13}C chemical shift to local modulations of the electronic density has made this technique one of the best probes of conformational aspects. In particular ^{13}C CPMAS (cross polarization magic angle spinning) appears to be well adapted for studying poly- (or poorly) crystalline solids, revealing qualitative

and quantitative features such as identification of phases and structural changes in crystalline polymorphs of pharmaceutical molecules (92-94). Recently the development of proper analysis methods to measure relative amorphous and crystalline fractions has raised considerable interest (90,95) and Lefort *et al.* have shown in a study of ball milling trehalose, that an NMR approach can be readily implemented in many situations involving continuous transformations of pure compounds and can still remain a successful method for estimating amorphous content of a sample, even though DSC might fail at it (10). Furthermore, Bøtker *et al.* utilized ss-NMR to explain the influence of different times of cryo-milling on the amorphization of indomethacin (81) (Fig. 5).

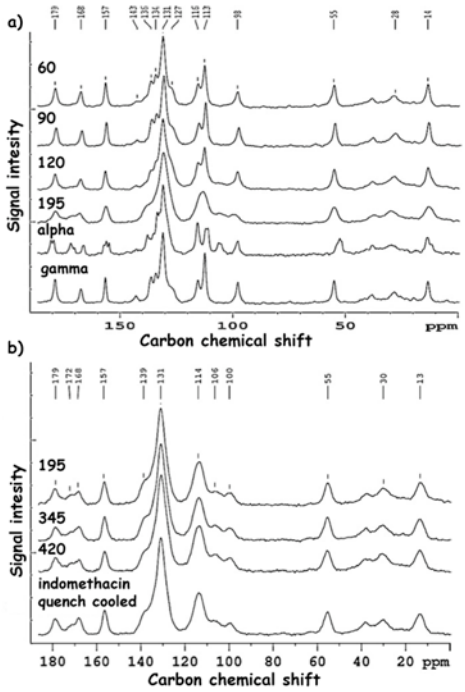

Fig. 5. a.) NMR spectra of 60,90,120,195 min cryo-milled samples of γ-indomethacin and the α-indomethacin. b.) NMR spectra of 195,345 and 420 min cryo-milled samples of γ-indomethacin and quench cooled amorphous indomethacin. The quench cooled sample and the cryo-milled samples with increasing time of milling show much broader peaks, which is the characteristic of amorphous substances (81). Adapted from ref. 81 with permission.

X-ray powder diffraction

The principle behind XRPD experiments is the random orientation of crystals in a substance. If the powdered crystals are randomly oriented, then for all sets of planes

some of the crystals in the powder will be in the right orientation with respect to the x-ray source to satisfy Bragg's law for the proper angle θ. What follows is that at least a few of the mineral grains in the powder will diffract for each of the planes during a scan through the angles θ. The more finely ground the powder is, the more likely it is that all orientations are sufficiently present. The whole XRPD method is based on the fact, that ideally every possible crystalline orientation should be represented very equally in a powdered sample. Two main types of powder diffraction experiments are possible: automated powder diffractometer experiments yielding (digital) computer output and Debye -Sherer experiments providing (analog) film output, utilizing a camera. A strip of film is wrapped around the powder sample so that diffracted beams from a fixed x-ray source can be recorded for all values of θ simultaneously. During the measurement the powder diffractometer moves both the x-ray source and an electronic detector through arcs of θ values and sends to a computer periodic signals proportional to the averaged diffracted x-ray intensity. Both experiments provide the intensities for diffracted beams as a function of the diffraction angle θ (or 2θ). The acquired data is then processed by the Rietveld method in order to minimize the residual function using a non-linear least squares algorithm. With that we can then refine the crystal structure of a compound. XRPD is typically used to determine the occurrence of a non-crystalline solid form, since it can be determined by observing the loss of the distinct XRPD peaks characteristic of crystalline order, and the appearance of a general "halo" pattern (1). There are however, a number of different non-crystalline phases that can give broad halos in the measured XRPD data, the most commonly observed for which are super-cooled liquids and glasses (96). Grinding or milling crystals can remove all traces of crystalinity according to XRPD. Cryo-grinding studies provide an ideal experimental approach to investigate the formation of amorphous material and the nature of the X-ray diffraction response. The typical behaviour observed when grinding a crystalline organic material to produce amorphous material is that an increasing percentage of the crystalline material will collapse to amorphous as a function of grinding time. The amorphous local packing generates broad halos in the XRPD pattern that are not correlated to the crystalline peaks. If no significant change is observed in the crystalline diffraction peaks upon grinding, the ground sample can be modeled as a phase separated binary mixture of thermodynamic amorphous and crystalline materials. Although successive

micronization should eventually lead to an amorphous structure, a possibility exists that the material has achieved a microcrystalline state, containing crystals so small they pass the detection of XRD. Johari *et al*. used DSC to distinguish between amorphous and microcrystalline states based on the presence or absence of glass transition when XRD failed to do so (97). It should also be noted that materials ground to or exhibiting the same X-ray amorphous pattern might crystallize at different times or to different crystalline forms. This can be perceived as a lack of control, resulting in amorphous materials that are not consistent. A better understanding of the short-range and long-range interactions in amorphous materials using atomic pair wise distributions can help explain and possibly help control the physical stability of the materials. Data analysis of X-ray amorphous patterns plays a significant role in addressing the primary regulatory concerns for drug commercialization: understanding, stability and reproducibility (98).

Atomic pair-wise distribution

The atomic pair-wise distribution function (PDF) is a method used to analyze the local structure based on the total x-ray scattering pattern from the crystalline, nano-crystalline, quasi crystalline or amorphous materials (99,100). The technique uses Fourier transformation of the XRPD diffractograms to produce a trace in the coordinate system. The y-axis in the PDF graph is corresponding to the probability of finding two atoms separated by a distance plotted on the x-axis. In the material science community the PDF technique has been used for several decades (101-105) and has more recently been applied by the pharmaceutical community to study short and long-range order amorphous glassy materials (106-111). It has been used in studies to investigate crystalline defects, help in the crystal structure determination (106,107,110) characterization of polymer/drug systems (111-113) and the use of multivariate data analysis has alleviated the interpretation of the PDF (108,111). It could be a possible route to gain a deeper insight into the degree of disorder in a milled sample. This is due to the fact that the PDF displays the probability of finding two atoms separated with a given distance (99). Therefore it could be expected that with increasing disorder in the sample, the signal amplitude of the PDF trace is further reduced, until the highest possible disorder for a given cryo-milling process has been achieved. Bøtker *et al.* have found that the PDF is capable of assessing the minimal

cryo-milling time that facilitates the highest degree of disorder and stability in cryo-milled samples of indomethacin (81).

Differential scanning calorimetry

Thermal methods have been used for amorphous content determination. DSC is a method commonly used for investigation of phase behavior of pharmaceutical solids, including quantification of the amorphous content (112). Depending on the instrument type, measure parameters and experimental conditions there are several methodologies that can be employed to determine the latter. Among them are conventional DSC, modulated temperature DSC (MTDSC) and hyper or high-speed DSC (HSDSC) (113). Conventional DSC is based on a linear heating rate. In temperature modulated DSC (TMDSC) a small sinusoidal temperature modulation is applied to the sample in addition to the usual linear ramp. In the newest technique, hyper-DSC, controlled fast heating and cooling rates of 50 up to 500 ºC/min are used (114). This significantly increases the measurment sensitivity because the increased scan rate leads to higher heat flow. Whereas amorphous character can be difficult to detect in highly crystalline solids using conventional DSC technique, hyper DSC can show glass transitions with much increased sensitivity and less time (114).

As mentioned before, amorphous materials exist as solid glasses or liquid like rubbers. The transition between these states is a second-order change phase, which occurs at T_g (115). There are at least three ways to determine T_g. Standard T_g is the temperature corresponding to the point on the heat flow curve where the specific heat change is 50% of the change in the complete transition. This is the temperature at which the heat capacity is midway between the liquid and glassy state (116). The glass transition can also be taken as the inflection point of the DSC curve associated with the glass transition. If a high relaxation peak follows the glass transition, the inflection point of the DSC curve can differ from the real inflection point of the glass transition. The fictive temperature refers to the point on the enthalpy curve where the change of slope occurs (117). The enthalpy curve is the integral of the specific heat curve (Fig. 6). The fictive temperature is the intersection of the extrapolated pre-transition and post-transition baselines on the enthalpy curve.

The ΔC_p is linearly proportional to the amorphous content in case that the amorphous glasses are in the same state. The largest change in the specific heat is equal to the

difference of crystalline and rubber states. When the glass transition is used for the quantification of the amorphous content, there has to be a reference material. A starting point for the development of this method is to ensure that the change of the specific heat of a 100% amorphous sample is reproducible. Many things influence ΔC_p. Glasses are known to change their properties when annealed at below their glass transition temperature. The release of the relaxation enthalpy that follows the glass transition corresponds to the enthalpy difference between the annealed and the quenched (non-annealed) glass (118). Karmwar et al. have shown that DSC curves of amorphous forms of indomethacin prepared by quench cooling and spray drying exhibit different T_g as shown in Fig. 6. and table II (8).

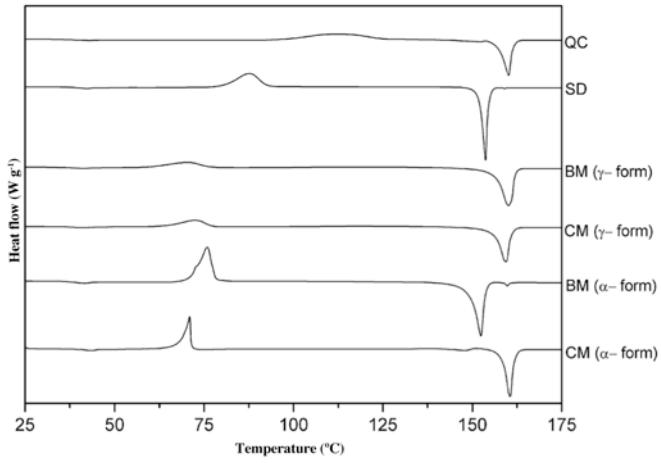

Fig. 6. DSC curves of freshly prepared amorphous form of indomethacin prepared by different preparative techniques. Key: QC- quench cooled, SD- spray dried, BM- Ball milled, CM- cryo-milled. Adapted from ref. 8 with permission.

Table II Thermal properties of amorphous forms of indomethacin prepared by different preparative techniques (8).

Technique	T_g(*C°*)	C_p at T_g(J/g°C)	Onset of crystallisation (°C)	ΔH_{relax}(J/g)
QC	42.15±1.16	0.50±0.003	96.93±1.02	1.03±0.26
SD	41.25±0.28	0.47±0.28	73.59±7.18	0.68±0.31
BM(γ-form)	39.23±2.19	0.57±0.03	62.14±4.16	0.28±0.09
CM(γ-form)	40.27±3.57	0.52±0.16	60.84±2.91	0.95±0.84
BM(α-form)	37.92±2.02	0.70±0.09	70.16±0.70	0.84±0.43
CM(α-form)	43.19±1.68	0.34±0.11	62.88±6.18	2.38±0.47

Mean SD values, n=3.

Solution calorimetry

Few publications have utilized solution calorimetry to determine the extent of drug and excipient crystallinity (119-123). It is a thermal technique in which the temperature change produced by a chemical or physical interaction during mixing of two solutions or of a solid or a liquid in a constant temperature environment is monitored as a function of time (120). Studies suggest that solution calorimetry can be used to determine the amorphous content of drug and excipient, when the solubility and dissolution rate of the compound in the chosen solvent are reasonably high. Typically 100% crystalline and 100% amorphous materials have been physically mixed to prepare samples of varying percent crystallinities, and a linear relationship between the heat of solution and the mass percent crystalline fraction present in the resulting mixture has been demonstrated (120,121). Harjunen *et al.* have shown that this method can also be used for assessment of amorphous content of lactose that was not completely dissolved in a solvent, which is an interesting find as a lot of pharmaceuticals and pharmaceutical excipients exhibit poor solubility. An excellent correlation was observed between the enthalpy of solution ($\Delta_{sol}H$) in water and the amorphous content of the samples as shown in Fig 7. Further, there was also a linear correlation between the enthalpy accompanied with an addition of a lactose sample to an over saturated aqueous solution ($\Delta_{sat}H$) and the amorphous content of the samples, as shown in Fig. 8. Therefore, solution calorimetry may represent a rapid and simple method for determining the amorphous content also in samples that are not completely dissolved in solvent (124).

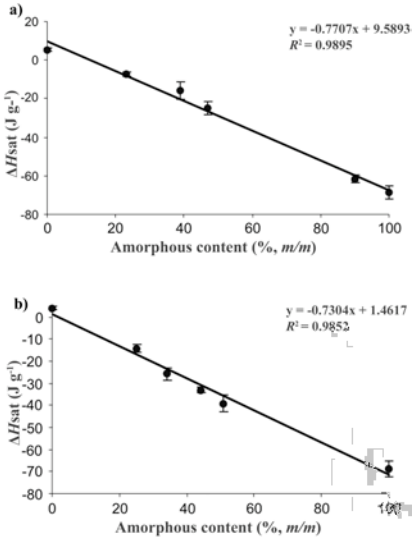

Fig. 7. (A) The relationship between the $\Delta_{sat}H$ and the amorphous content of physical mixtures. Mean values ± SD are shown (n=4). (B) Relationship between the $\Delta_{sat}H$ and the amorphous content of the spray dried samples. Mean values ± SD are shown (n=4). Adapted from ref. 124 with permission.

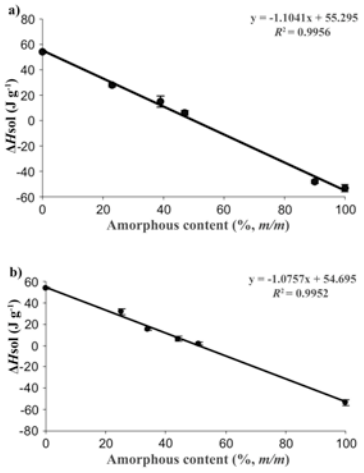

Fig 8. (a) Relationship between the $\Delta_{sol}H$ and the amorphous content of physical mixtures. (b) Relationship between $\Delta_{sol}H$ and the amorphous content of spray dried samples. Mean values ± SD are shown (n= 4-8). Adapted from ref. 124 with permission.

Density measurements

Solid density is a physical property the value of which is frequently required in both fundamental and applied pharmaceutics. True density may be obtained using pycnometry, flotation density measurement, or from single crystal structure. Flotation density measurement however, is not suitable for powder mixtures (125,126). Crystalline materials in general have a higher density than their amorphous counterparts because the atoms in the crystal lattice are located at a minimum possible distance from each other. An increase in lattice disorder (decreasing crystallinity) usually results in an increase in volume and therefore a decrease in density. The changes in crystalinity should therefore be accompanied by gradual, progressive changes in density (127). The degree of crystallinity of a sample can be determined by *Eq.* (*2*) (ϱ – obtained density, ϱ_a-density of the fully amorphous state, ϱ_c-density of the crystalline state.)

$$\%Crystallinity = \frac{\rho-\rho_a}{\rho_c-\rho_a} x100 \qquad (2)$$

Different density measurement techniques were used in literature to detect low levels of the amorphous phase in crystalline pharmaceuticals (128) or to determine the crystallinity of the sample (1,129). Therefore density measurements can also be used as an alternative technique to determine the solid state of pharmaceuticals. Saleki-gerhardt *et al.* have shown the increase of density correlates with the disorder of solid sucrose (Fig. 9) (130).

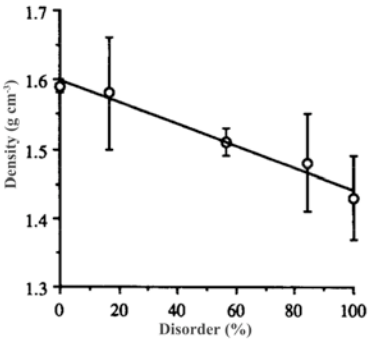

Fig. 9. Density vs. percent disorder for mixtures of amorphous and crystalline sucrose. Adapted from ref. 130 with permission.

Gravimetric vapour sorption

Gravimetric vapour sorption is a technique used to determine the vapour sorption isotherms. The instruments measure how the mass of the sample changes as the vapour environment surrounding the sample is altered. An increase in mass is typically associated with vapour sorption, whilst the occourance of mass decrease is attributed to vapour desorption. Mixed saturated and dry carrier gas streams are used in order to control the vapour concentration around the sample. Dynamic vapor sorption has previously been used to characterize amorphous or partially amorphous systems (131). The principle behind it is that amorphous materials typically have a higher surface area and vapour affinity than their crystalline counterparts. More recently Vollenbroek *et al.* have developed a method, which is based on gravimetric vapour sorption, that allows the determination of the amorphous lactose content over the range of 0.1-100% (132). However, it must be noted that using dynamic vapour sorption for determination of amorphous content may be flawed, because direct comparison of partially amorphous systems to wholly amorphous and wholly crystalline systems may result in significant different outcome, due to the fact, that semicrystalline materials exhibit different molecular mobility, and thus vapour sorption, when compared to wholly amorphous or crystalline material (13).

Inverse gas chromatography

Inverse gas chromatography (IGC) is a vapor sorption technique in which the powder is packed in a column and known vapors (usually at infinitine dilution in a carrier gas) are injected. It provides access to several physiochemical (surface and bulk) properties of materials, including their surface energy, phase transitions, solubility parameter, crystallinity, and acid-base characteristics (133,134). It has also been used to detect surface energy change caused by milling (135). For sucrose it was found that milling does not influence the particles crystal structure, but only the particle size and relative exposure of specific crystal planes. From the retention times of the probes it is possible to assess the surface nature of the material in the column (130). It can be expected that this vapor sorption approach is also able to detect small amounts of amorphous content (88). Planinsek *et al.* have shown that IGC is an efficient method for the quantification of the fraction of amorphous surface of milled indomethacin. It

was shown that the combination of IGC with DSC enables not only quantification but also the localization of structural changes of milled indomethacin. That is, it enables differentiation between the transformed structure at the surface of the particles and transformations of the bulk region (136).

MID infrared spectroscopy

Middle (MID) infrared methods can reflect significant spectral differences between crystalline and amorphous phases and hence are used to quantify the crystalline content, as the intensity of the vibrational bands is directly proportional to the concentration of the concerned phase. Amorphous forms of a given drug give rise to IR spectra that differ from their crystalline counterparts. The origin of these differences relate to both the wider range of conformations typically present in an amorphous solid, which normally leads to the presence of broader peaks relative to those found in the crystalline spectrum, as well as differences in intermolecular interactions (1). Infrared procedures for measuring the degree of crystallinity are based upon the measurement of intensity of a peak, which is characteristic of the crystalline state with reference to a peak, which is independent of the crystal state of the substance. Nakai *et al.* have shown that it is possible to study the effect of grinding on the crystallinity of microcrystalline cellulose (MCC) by infrared technique (75) and similarly, Otsuka and his coworkers also studied the effect of grinding on the crystallinity of cephalothin (137). MID-IR has also been found to be extremely useful to study amorphous solid dispersions (138). A study by Tang *et al.* used FT-IR spectroscopy to characterize differences between crystalline and amorphous phases of dihydropyridine calcium channel blockers. For all compounds, the amorphous and crystalline samples gave rise to different spectra (139).

Near infrared spectroscopy

Near infrared spectroscopy (NIR) is a noninvasive technique, which requires no sample preparation; it is also non-destructive enabling complete sample retrieval especially if used with diffuse reflectance option. In addition further quantitative information can be extracted from the data using chemoinfometrics, where the collected data is processed by means of statistical and applied mathematical techniques. Usually, quantification of crystallinity is performed using the first (140)

or second derivate spectra (95,141,142). Physical and chemical information may be obtained such as polymorphism (143) and mutarotation (144). Buckton *et al.* (145) have shown that it is possible to monitor the crystallization of amorphous lactose in real time though examination of NIR spectra at certain wavelengths. Moreover Hogan *et al.* have shown that it is possible to quantify the amorphous content of lactose with NIR (144). Luner *et al.* used the technique to determine the crystallinity of several pharmaceuticals including indomethacin, lactose, ampicillin and sucrose (141). NIR was also used by Otsuka *et al.* to monitor the stability of amorphous indomethacin in humidity controlled 96-well plates (146). Furthermore, Otsuka *et al.* have shown that the crystallinity of unknown samples obtained by FT-NIR chemoinfometrical spectrometry was consistent with that obtained by conventional X-ray powder diffractometry and was more accurate (Fig. 10). According to the change in NIR absorbance of indomethacin, the solid structure of amorphous indomethacin was significantly different from that of the crystalline form (147).

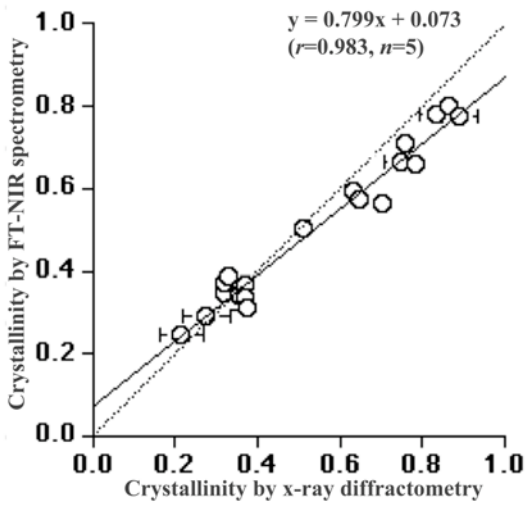

Fig. 10. Relation between predicted crystallinity of unknown indomethacin samples obtained by conventional X-ray powder and FT-NIR method. Bars represent standard deviation. Adapted from ref. 147 with permission.

Raman spectroscopy

Raman spectroscopy is a spectroscopic method used to observe vibrational, rotational, and other low-frequency modes in a system. It probes properties of the molecule itself, and changes in the solid-state properties of a substance are inferred from

changes in the molecular conformation and molecular environment. This is due to different packing conditions of the molecules in the different solid forms. Differences can then be seen as subtle changes in the peak positions and intensities in the Raman spectra (148). It has recently been used in various studies for means of differentiation between differently prepared amorphous forms of the same substace. Karmwar *et al*. have shown that it is a suitable method for the detection of differences between differently prepared amorphous forms of indomethacin and simvastatin (8,149). Similarly, Zimper *et al.* have shown that Raman spectroscopy combined with multivariate analysis can detect and quantify disorder in both indomethacin and simvastatin. Nevertheless the authors point out that raman spectroscopy as a molecular level technique is sensitive to the near order of solid materials and therefore could 'underestimate' the degree of disorder if the material remains near range ordered to a certain extent (*i.e.*, exists as a dimer) in the amorphous state (79). However, in contrast to the findings of the previous authors, Boetker *et al.* have found that it is unable to distinguish between differently prepared samples of amlopidine (150).

Dissolution tests

As the molecular mobility of the amorphous form is higher when compared to the equivalent crystalline form it may have enhanced dissolution rate. This difference can be used to estimate the degree of amorphous content in a given sample. Although the amorphous form will have a higher dissolution rate because of high-surface free energy, there is an inherent risk of devitrification in the dissolution fluid (151), rendering dissolution tests useless for the characterization of the solid state. However, the amount dissolved has been used to quantify the crystallinity in case of amorphous solid dispersions. (152). The main problem encountered in this technique is the effect of surface area; if not controlled stringently it can significantly affect results. For controlling the surface area, the powder is compressed. This may lead to probable phase changes. Also the dissolution medium needs to be carefully selected as the components of the medium can influence the final outcome (88). Care must be taken using this method if the transformation process includes extensive crystal defect formation, which is especially expected in the early stages of the amorphization due to mechanical milling. Because the dissolution depends on solvent accessible surface

and surface energy crystal defect formation and increased surface could lead to false results.

Microcalorimetry

Microcalorimetry is a technique that has attracted much interest among pharmaceutical researchers as it can be used for various studies of preformulation. One the possible uses of microcalorimetry is also the assessment of the amorphous content (153-156). The determination of the amorphous content by microcalorimetry is based on the fact that the conversion from the amorphous to the crystalline form is detected as an exothermic heat flow. The area under the exothermic peak is then proportional to the amorphous content. In some cases the sensitivity of the calorimetric approach was even proven to be better of the XRPD. Furthermore, one of the advantages of the technique is that it allows to measure the "real time" response in the calorimeter monitoring the recrystallization of the amorphous substance (154).

Terahertz spectroscopy

The detection of terahertz radiation waves (THz), which have a frequency of between 0.1 and 10 THz, is potentially very useful in probing intermolecular-level long range without sample contact and destructive treatment when characterizing solid-state materials, since it can induce low frequency bond vibrations, crystalline phonon vibrations, hydrogen-bonding stretches, and torsion vibrations (153). A recent study by Otsuka *et al.* has shown that THz spectroscopy is suited as a discrimination method between different amorphous states of pharmaceuticals called poly-amorphous solids". Fig. 11 shows the THz spectra of the polymorphic crystalline forms and the amorphous solids of indomethacin obtained by the transmittance method. (9).

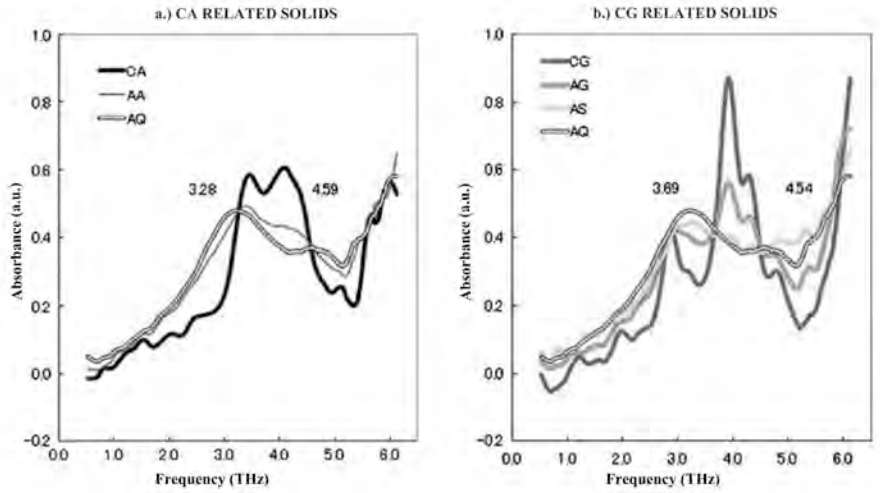

Fig. 11. The terahertz spectra of amorphous samples derived from the α -form and γ -indomethacin using different preparation techniques. B) powder samples with γ -form; A) powder samples prepared from α -indomethacin. CG- γ -indomethacin, CA α -indomethacin, AQ - fast cooled amorphous indomethacin, AG - Ground amorphous γ -indomethacin, AA α -indomethacin, AS - slowly cooled indomethacin, IMC A - cryo milled α -form indomethacin and IMC G - cryo milled γ -form indomethacin (9). Adapted from ref. 9 with permission.

CLARITHROMYCIN

IUPAC: 6-(4-dimethylamino-3-hydroxy- 6-methyl-tetrahydropyran-2-yl) oxy-14-ethyl-12,13-dihydroxy- 4-(5-hydroxy-4-methoxy-4,6- dimethyl-tetrahydropyran-2-yl) oxy-7-methoxy-3,5,7,9,11, 13-hexamethyl-1- oxacyclotetradecane-2,10-dione

Molecular formula: $C_{38}H_{69}NO_{13}$

Molecular weight: 747.96 g/mol

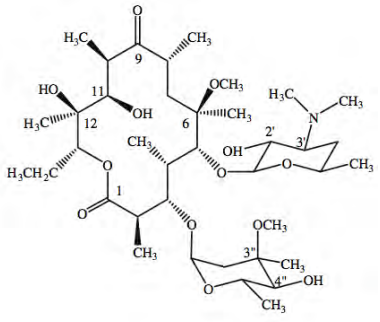

Fig. 12. Structural formula of Clarithromycin

Clarithromycin is a semi synthetic broad-spectrum macrolide antibiotic, which exhibits excellent activity against gram-positive bacteria, some gram-negative bacteria, anaerobic bacteria, mycoplasma, and Chlamydia (158).
As it is a macrolide it reversibly binds to the domain V of 23S ribosomal RNA (rRNA) of the 50s subunit of the bacterial ribosome inhibiting RNA-dependent protein synthesis (159, 160). One of advantages Clarithromycin has compared to other macrolide antibiotics, such as erythromycin, is that appears to be completely and reliably absorbed from the gastrointestinal tract (161). This is due to the greater acid stability (162). The overall bioavailability of a 500 mg oral dose of Clarithromycin is 55% with a C_{max} of 2.1-2.4mg/L, t_{max} of 2h, and an AUC of 19 mg/L*h.
Clarithromycin is metabolized to an active metabolite, 14-hydroxyclarithromycin. The drug is principally excreted via the liver and kidneys (163). Larger doses of clarithromycin result in nonlinear increases in the $t_{1/2}$ and in the area under the plasma concentration-time curve (AUC) of clarithromycin because of saturation of the metabolic pathway (164).

Clarithromycin is a white to off white crystalline powder. It is soluble in acetone, slightly soluble in methanol, ethanol, and acetonitrile, and practically insoluble in water. This along with a good permeability classifies it as a Biopharmaceutical class II drug.
Polymorphism of clarithromyicin has been reported in patents and three crystal modifications of clarithromycin “Form I”, “Form II” and “Form 0” were characterized (165-167).

AIM OF THE WORK

The aim of this study is to prepare the amorphous form of CAM by different means of amorphisation (spray-drying, cryo-milling, quench-cooling). Once suitable methods of amorphisation are developed the prepared amorphous samples are to be compared among each other and to both crystal form I and II of CAM using means of solid-state amorphisation. X-ray powder diffraction, solid-state nuclear magnetic resonance, differential scanning calorimetry and attuned total reflection infrared spectroscopy are to be employed for the screening of differences. In addition to that, the increase of the

amorphous fraction will also be determined in both CAM form I and II samples, milled for different time intervals.

MATERIALS AND METHODS

MATERIALS

Substances:

- Chlaritomycin Form II (Purity 99%, Krka d.d, Slovenia)

Solvents:

- Tetrahydrofuran (Merck, Germany.)
- Methanol (Sigma Aldrich, Germany)
- Ethanol 96% (Sigma alrdrich, Germany)

Laboratory equipment:

- Measuring cylinder
- Volumetric flask
- Florence flask
- Metal tongs
- Beaker
- Watch glass
- Metal spatula

Apparatus:

- Analytical weight scale Mettler Toledo AG 245, Switzerland
- Weight scale A&D Instruments LTD, GH-300-EC, Italy
- Vibrational Mill Pulverisette 0, Fritsch, Germany
- Differential Scanning Calorimeter DSC1, Mettler Toledo, equipped with STARe Software v9.30, Switzerland
- Varian NMR system equipped with a 3.2 mm Varian probehead
- X'Pert PRO MPD diffractometer (PANalytical)
- Nicolet Nexus FTIR Spectrometer (Nicolet Instrument Co., Madison, USA) equipped with a DTGS (Desaturated Triglycine Sulphate) detector.

Spectral data were acquired with Omnic E.S.P. software version 5.2 (Nicolet instrument Co.)

- Büchi Mini Spray Dryer-290 (Büchi Switzerland)
- Thermogravimetric analyzer Mettler-Toledo, TGA, equipped with STARe Software v9.30, Switzerland
- Büschi Rotavapor R-114, Switzerland

METHODS

SAMPLE PREPARATION

Preparation of Clarithromycin polymorphs

The form II of clarithromycin was used as received. Form I was obtained by dissolving the appropriate amount of form II in THF at 66ºC and then cooling it down to initiate precipitation. The solvent was then removed by evaporation under a vacuum.

Three transformation routes were used to prepare amorphous clarithromycin samples: melt quenching, spray drying and cryo-milling.

Melt quenching

Clarithromycin (Form II and I) (30mg) was melted at 230ºC using alumina pans (volume 150 µl) in a Mettler-Toledo TGA (Mettler Toledo, Switzerland) and then cooled immediately by placing the sample in liquid nitrogen. Melting was monitored with DSC analysis to prevent thermal degradation of the sample. Process was repeated until high mass of amorphous sample was collected. Prepared samples were stored at 6ºC in airtight vials.

Spray drying

Clarithromycin (form II) (1g) was dissolved in 100ml methanol (99%). The resulting 1% (m/v) clarithromycin solution was spray dried under nitrogen atmosphere (600kPa) using a Büchi Mini Spray Dryer-290 (Büchi Switzerland). A

dual-fluid nozzle with a 0.7 mm nozzle tip and a 1.5 mm nozzle cap was used to atomize the solution in the cooling chamber. The original glassware of the apparatus was used, consisting of the process chamber (15 cm diameter and 60 cm length), a high-performance cyclone, an aspirator, and an outlet filter. The inlet temperature was 90ºC; the aspirator was set to 100% and the pump to 10%. This was repeated several times and the obtained product was stored in airtight glass vials at 6ºC.

Cryo-milling

Amorphous clarithromycin was prepared from both the II and I form of the substance. The material was milled using an oscillatory ball mill Pulverisette 0 (Fritsch, Germany, Fig. 13) at 2/3 of its maximum frequency. The sample powder (1g) was placed in a stainless steel mortar and milled with a stainless steel grinding ball provided by the manufacturer. Both forms were milled at 6ºC with an appropriate frequency at different time intervals. Samples were collected and stored in airtight glass vials at 6ºC.

The time intervals in which the samples were taken for both substances were, 15min, 30min, 1h, 2h, 4h, 24h, 48h.

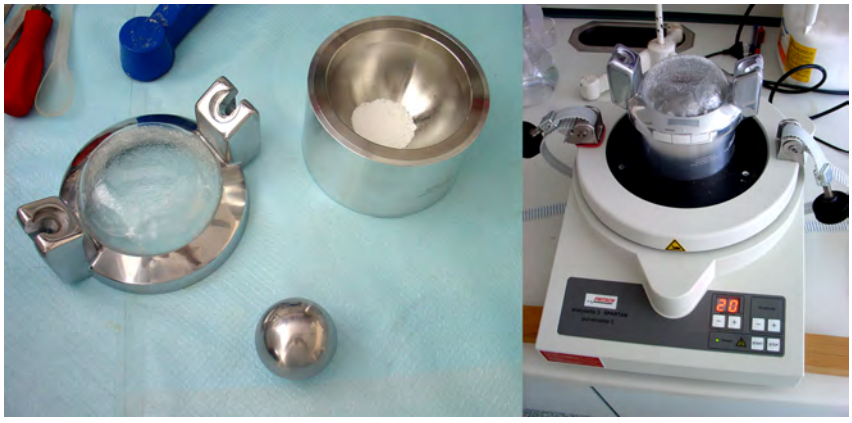

Fig. 13. Oscillatory ball mill Pulverisette 0.

CHARACTERISATION OF SAMPLES

Scanning electron microscopy (SEM)

The morphological properties of the obtained samples and the internal structure characteristics were evaluated using a scanning electron microscope (SEM) Supra^{TM} 35VP (Zeiss, Germany).

X-ray powder diffraction

X-ray powder diffraction (XRPD) patterns were collected on a Siemens D-5000 diffractometer using CuK_{α} radiation. The samples were scanned at a range between 2 and 40º 2θ at step 0.04º 2θ with an integration time of 1 s.

Differential Scanning Calorimetry (DSC)

DSC studies were performed using Mettler-Toledo DSC 1 (Mettler Toledo, Switzerland) instrument equipped with an intracooler. The instrument was calibrated for temperature and enthalpy using indium. The samples were hermetically sealed in an aluminum pan and heated at a constant rate of 10 K/min, over a temperature range of 0—240°C. An empty aluminum pan was used as a reference. Inert atmosphere was maintained by purging nitrogen gas at the flow rate of 50 ml/min. Sample weights were in the range of 2-6 mg.

Attenuated total reflectance (ATR) Fourier transform infrared spectroscopy

A Nicolet Nexus FTIR Spectrometer (Nicolet Instrument Co., Madison, USA) equipped with a DTGS (Deuterated Triglycine Sulphate) detector was employed for all experiments. A diamond ATR accessory (DuraSample IR – Technologies Danbury, USA) was employed for ATR FTIR experiments. Each sample was sampled 3 times and their IR spectra averaged. The same pressure was used for all measurements. Each spectrum comprises 1000 co-added scans measured at a spectral resolution of 4 cm^{-1} in the 4000–600 cm^{-1} range with an aperture of 36.

Spectral data were acquired with Omnic E.S.P. software version 5.2 (Nicolet instrument Co.). Baseline and ATR corrections were used for processing the spectra before quantitative analysis.

Solid-state nuclear magnetic resonance

^{1}H MAS (Magic-Angle Spinning) and ^{1}H-^{13}C CPMAS (Cros-Polarization Magic-Angle Spinning) NMR spectra were recorded on a 600 MHz Varian NMR system equipped with a 3.2 mm Varian probehead. Larmor frequencies for ^{1}H and ^{13}C were 599.72 MHz and 150.82 MHz, sample rotation frequencies were 20 kHz and 16 kHz, and the numbers of scans were 64 and 600, respectively. Repetition delays between scans were 5 s in all experiments. ^{1}H 90° excitation pulse was 2 μs. ^{1}H-^{13}C CPMAS experiment employed RAMP[ref1] during CP block and high-power XiX heteronuclear decoupling during acquisition. Chemical shifts of ^{1}H and ^{13}C signals were referenced to the corresponding signals of tetramethlylsilane (168, 169).

RESULTS AND DISCUSSION

Visual characteristics

Cryo-milled clarithromycin samples, which were taken at different time intervals, were white powders with no distinctive features, the quench-cooled clarithromycin was of glass looking structure with no signs of degradation and the spray dried sample was a white powder with a very aerous structure.

SEM

SEM was used to obtain more information about the surface and morphology of CAM samples (Fig. 14.). Both crystal form I and II presented particles with a diameter ranging from 250-500μm. The surface of the form II sample appeared to be more textured than that of the form I. The CM samples of the crystal forms presented particles with a smooth surface, the diameters of the particles raging from 50-200μm. The SD sample were fused spherical particles with a diameter around 10μm and a

highly porous surface. The QC sample presented particles, which were larger than 500µm and had a flat surface.

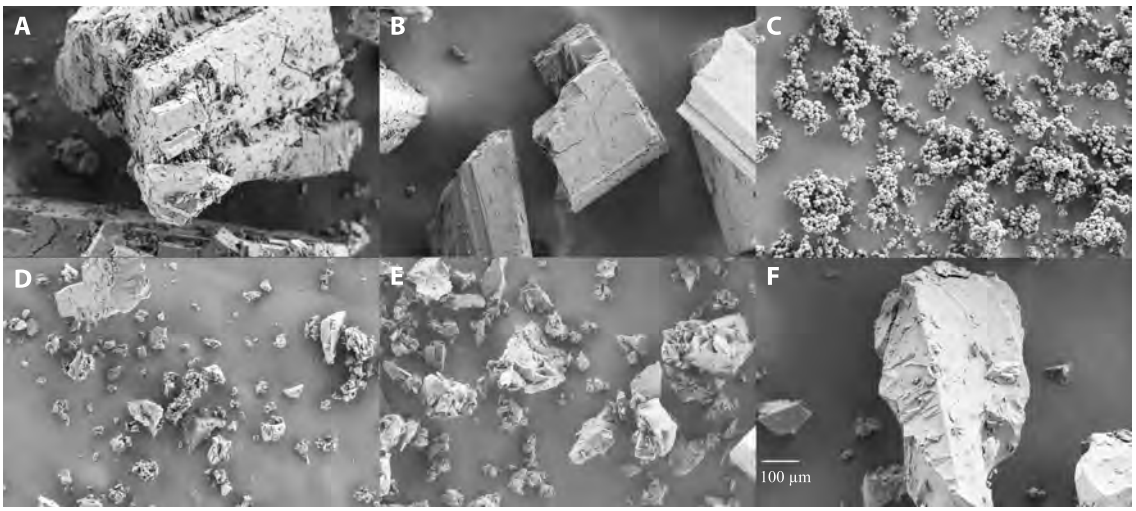

Fig. 14. SEM images of A) CAM Form II, B) CAM Form I, C) CAM Form II CM 72h milled, D) CAM Form I CM 72h milled, CAM SD, CAM QC. Magnification 1000x.

X-ray powder diffraction measurements

Both crystal forms I and II of CAM (CAM) presented typical CAM crystalline XRPD spectra Fig. 15 (170). Quench-cooling, spray drying and long enough cryo-milling of crystalline forms I and II of CAM resulted in products that were X-ray amorphous (amorphous halo, absence of crystalline peaks) or partially amorphous. Cryo-milling at 6ºC resulted in products, which were amorphous or partly amorphous depending on the time of milling. However, recent literature suggests that even though XRPD spectra present no crystalline peaks, very small crystals can avoid being detected by XRPD screening (97). The XRPD spectra of amorphous CM, QC and SD samples of CMC do not differ from each other, all showing spectra with relative broad and flat maxima around 12.0 and 18.5 º2θ. This suggests that XRPD would not be a suitable method to detect differences between differently prepared amorphous samples if they exist. Furthermore, differences in time needed for complete amorphisation were observed between CM samples of CAM forms II and I. CAM Form I appearing to be fully XRPD amorphous after 2 hours of milling time and CAM form II after 10 hours. No changes were observed when the samples were milled for additional 62 hours. In

addition to those findings, the disappearance of the crystal form I signature peaks was observed at the shortest milling intervals for CM CAM Form I samples. After the CAM form I sample was milled for 30 min the spectral peaks seen in the XRPD spectra resembled those typical for CAM form II (Fig. 16). This could indicate a transformation of the less stable form I to the more stable form II, which is further backed up by the DSC measurements and ss-NMR data.

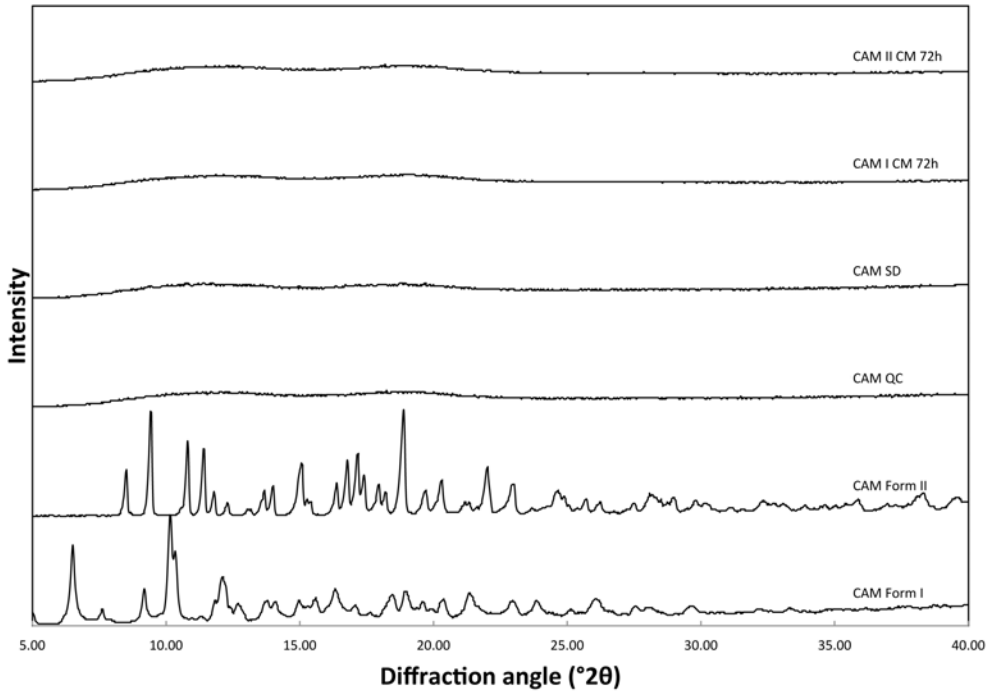

Fig. 14. XRPD spectra of CAM Form I, CAM Form II, CAM I CM – 72h, CAM II CM – 72h, CAM SD, CAM QC

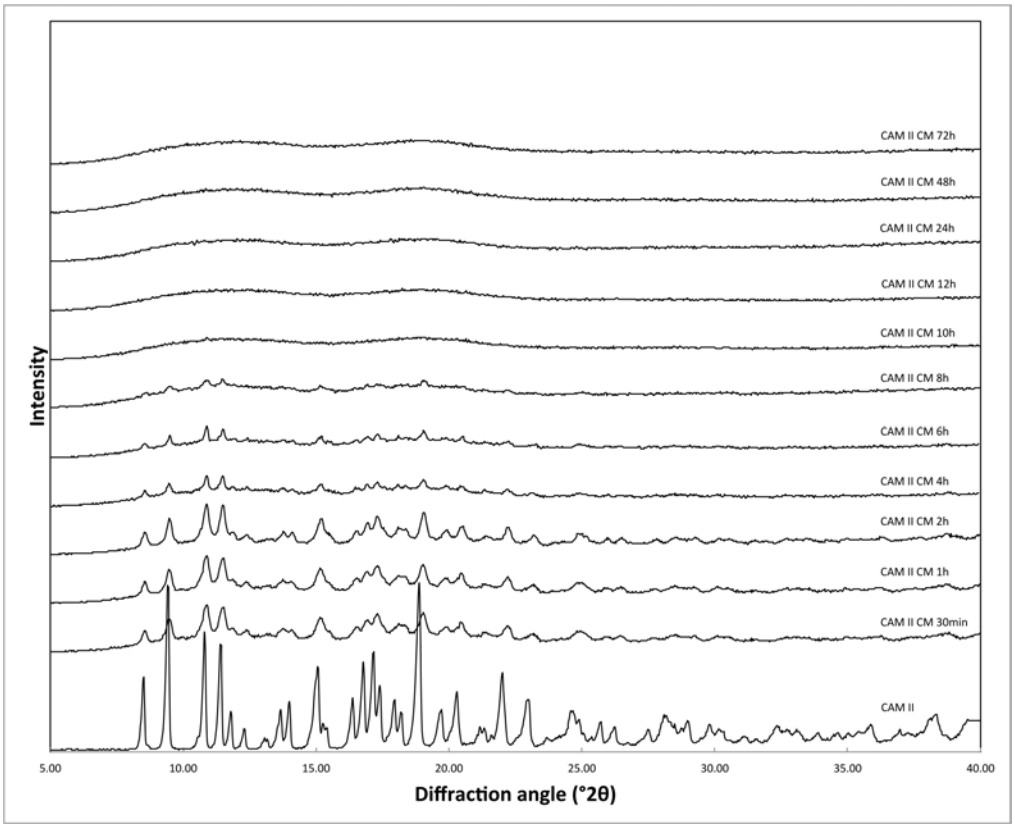

Fig. 15. XRPD spectra of CAM form II CM for 15min, 30min 1h, 2 and, 4 and 72h.

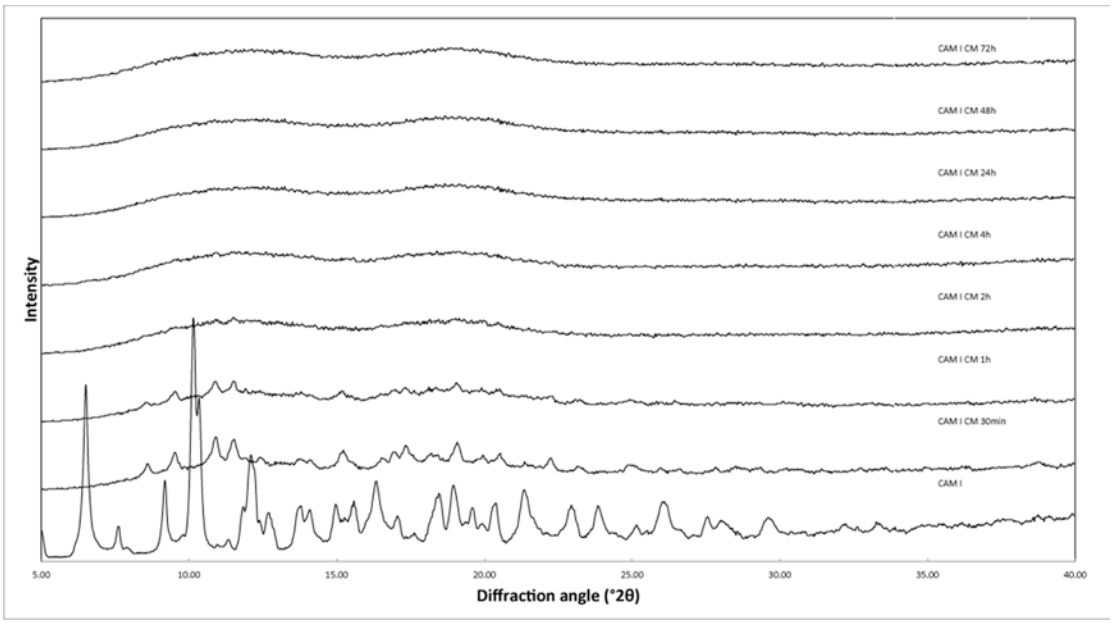

Fig. 16. XRPD spectra of CAM form I CM 30min, 1h, 2h, 4h, 24h, 48h, 72h.

DSC measurements

DSC measurements showed that both crystal forms II and I exhibit an endothermic peak with the onset at 229ºC, which was attributed to melting (Fig. 17). Crystal form I showed an additional peak with the onset at 119ºC, which was attributed to the transformation of the less thermodynamically stable form I to the more stable form II. The DSC curves of all cryo-milled samples of CAM taken at times of milling longer than 30min, exhibited a slight change in heat capacity (ΔC_p) at around 110-120 ºC (Fig. 17), followed by an exhotermic event in the range of 125-130ºC and a subsequent endothermic peak at 229ºC. Samples taken before 30 min of milling time resembled predominantly the curves of pure form I or II depending on which crystal form was milled. The change in heat capacity can be attributed to the glass transition (T_g) of the sample and the exothermic event (T_c) was associated with recrystallization. In contrast the SD and QC samples exhibited a change in heat capacity associated to glass transition in a much smaller extent, although they exhibited the same exothermic event in the range of 125-130ºC and the endothermic peak at 229ºC as the milled samples did (Fig. 17, Fig. 18)

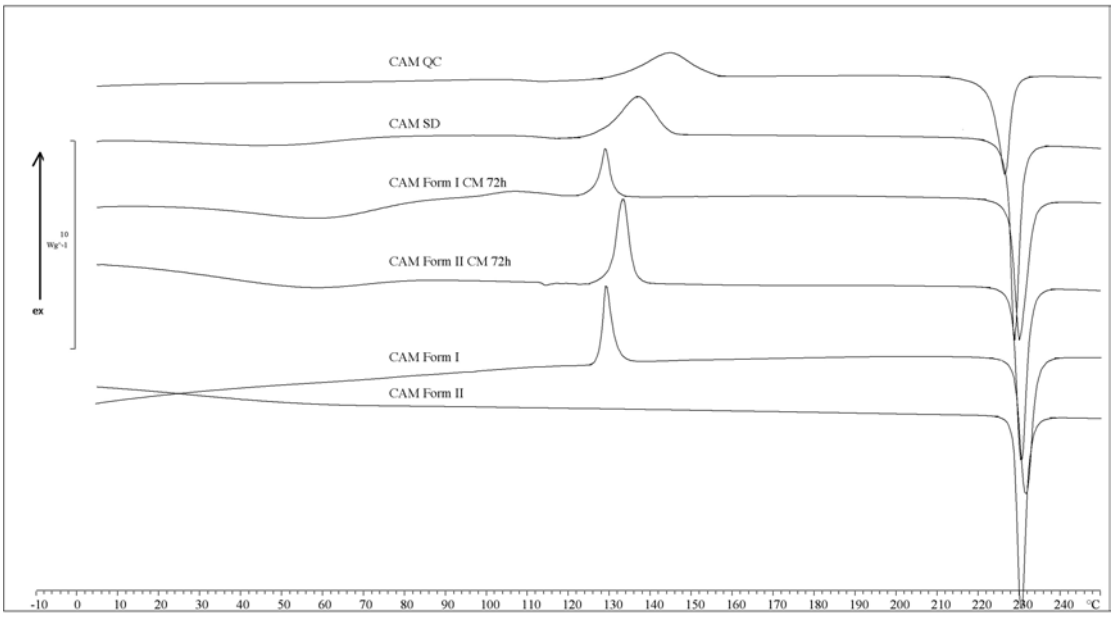

Fig. 17. QC, SD, CM Forms I and II forms of CAM and the form (II) and (I) of CAM.

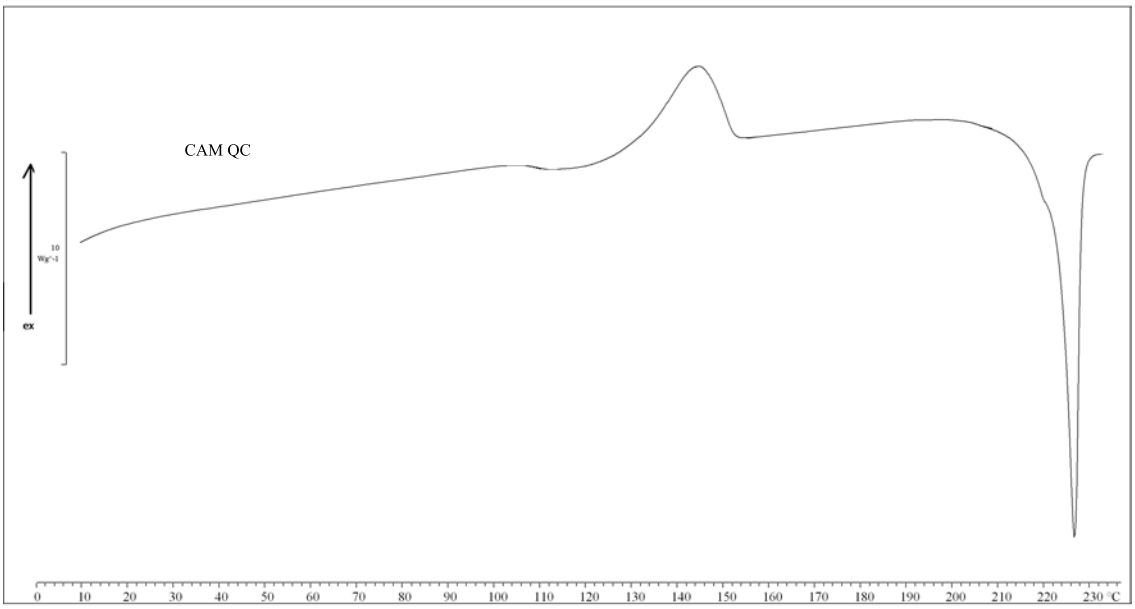

Fig 18. DSC Curve of QC CAM

Solid state nuclear magnetic resonance

Solid-state nuclear magnetic resonance (ss-NMR) was utilized to further investigate the influence of different amorphization methods on CAM structure. The differences were seen in the changes of spectral peaks at 220, 180, 110, 80-0 ppm. As seen in Fig. 19 the spectra of the CM form II and I CM 72h, QC and SD, samples are similar to the pure form II spectra, although presenting much broader spectral peaks. The broadness of spectral peaks of CM CAM Form II increases with time of milling, although there is no further broadening of the spectral peaks after 24h time of milling., indicating full amorphisation. The ss-NMR spectra indicate that full amorphisation is achieved by quench cooling, spray drying and cryo milling after 48h time of milling (Fig. 20). It appears that the CAM QC, CAM CM 72h milled and CAM SD spectra do not differ among each other.

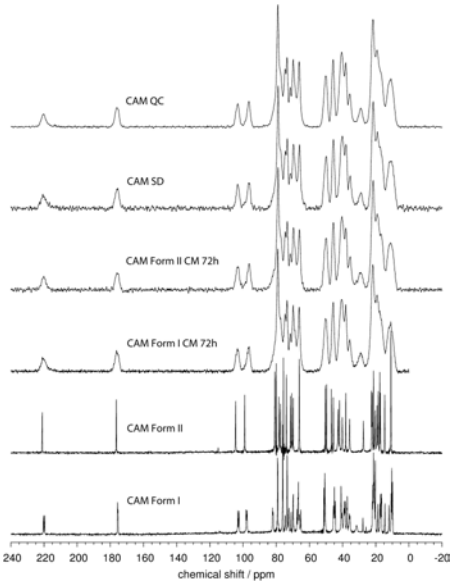

Fig. 19. ss-NMR spectra of CAM Form I, CAM Form II, CAM Form I CM 72h, CAM Form II CM 72h, CAM SD, CAM QC.

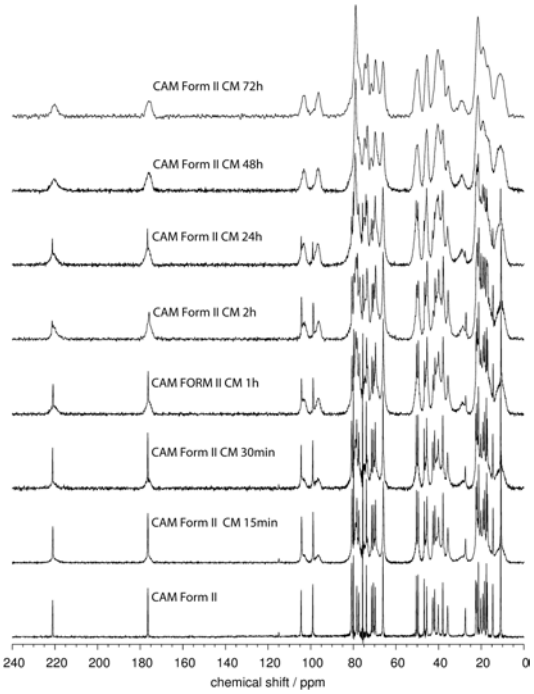

Fig. 20. ss- NMR spectra of cryo milled CAM form II samples.

SS-NMR was also utilized for the quantification of the amorphous fraction in cryo-milled samples of form II. The percentage of the amorphous fraction was determined by integration of spectral peaks around 221 ppm, since they were more consistent when compared to other ss-NMR spectral peaks of CAM. The evolution of the amorphous fraction as function of the milling time is given in Table III. The results

clearly indicate a graduate increase of the amorphous fraction with the time of milling. For the CM Form II full amorphisation is achieved after 24 hours of milling time. Initial quick increase in the amorphous fraction can be seen, which is then followed by a gradual amorphisation with time. A similar amorphisation kinetic was detected by ss-NMR in milled lactose by Caron et al. (80).

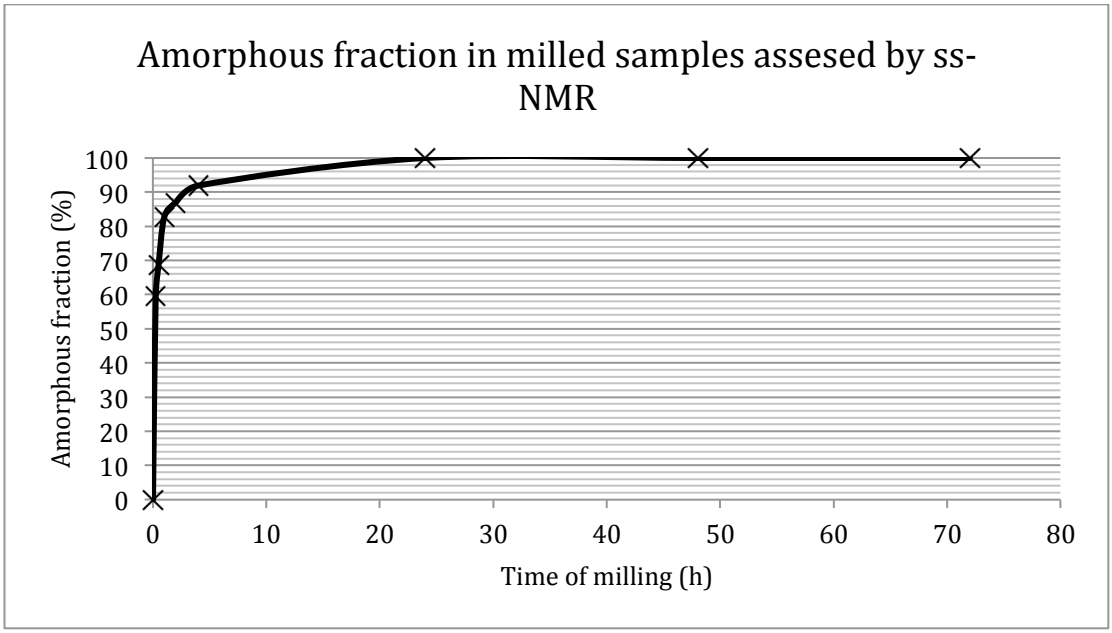

Fig. 22. The assessed percentage of the amorphous fraction as a function of the time of milling by ss-NMR.

ATR measurements

The ATR spectra of all the freshly prepared amorphous samples differ from the crystalline samples (Fig. 21). Furthermore, the AT-IR spectra of the QC sample differed significantly from the SD and CM spectra, presenting no peak at 1200 cm^{-1}. Significant differences in the spectra between the crystal form I, II and the amorphous forms could be noted at different wavelengths. Both crystal forms present two spectral peaks in the spectrum at 600 cm^{-1}, whereas the amorphous samples present only one. In contrast to CAM form II QC,SD and CM samples of form I and II milled for more than 24h do not show spectral peaks at 1100 cm^{-1} and 1430 cm^{-1} (Fig. 23).

The gradual disappearance of signature spectral peaks of the crystal form I and II at 600 cm^{-1}, 1100 cm^{-1} and 1430 cm^{-1} can be seen with increased time of milling of both

crystal forms. However, the time needed for the peak disappearance (full amorphisation) does not correlate for form II and I. The faster amorphisation of form I correlates with the ss-NMR and XRPD measurments. Furthermore, the peak at 600 cm^{-1} does not seem to disappear even when the crystal form I is milled 72h, as opposed to the IR spectra of form II, where the peak disappears after 1h of milling time (Fig. 24).

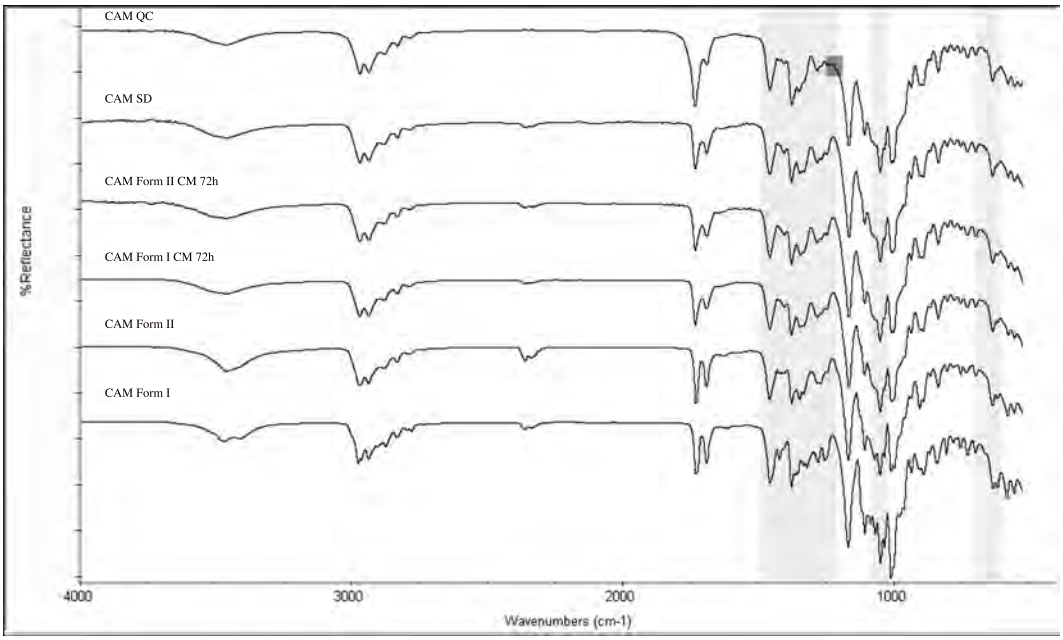

Fig. 23. ATR spectra of CAM form I, form II, CAM Form II CM 72h, CAM Form I CM 72h, CAM SD, CAM QC.

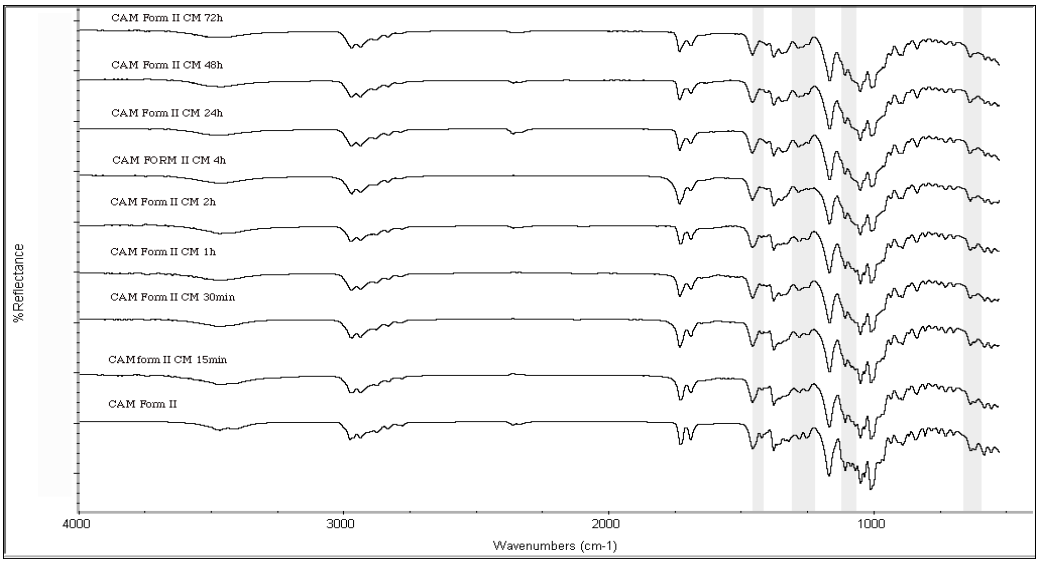

Fig. 24. CAM Form II milled for different time intervals.

CONCLUSION

Due to their higher solubility and bioavailability in comparison to the crystalline state amorphous solids have received a lot of attention in the past decades. In this research we have used three methods of preparation of amorphous solids and their characterization. There is a wide array of solid-state charactersiation techniques that can be utilized to investigate the possible different properties of the amorphous forms of substances prepared by various methods is also presented, as amorphous forms prepared by different means of amorphisation often exhibit differences in stability and solubility. The solid-state characteristics of three differently prepared amorphous (cryo-milled, quench-cooled and spray dried) and two crystal forms of CAM were investigated in this research. The data obtained by DSC, ss-NMR, XRPD measurements shows that, although these are all methods suitable for the differentiation between the amorphous and crystalline state, in case of chlaritromicin any differences between the amorphous forms of samples prepared by different methods were detected. Among the samples that were assumed to be amorphous SD and CM CAM samples presented the glass transition temperature event in a much smaller extent when compared to the QC CAM sample. However the QC sample did not significantly vary in crystallisation temperature and melting point when compared to the SD and CM CAM samples. ss-NMR failed to detect any difference between the prepared amorphous samples, however we have shown that it is a suitable method for the assessment of the amorphous fraction for samples milled for different intervals. There was a clear correlation between milling time and the percentage of the amorphous fraction in the cryo-milled samples. Furthermore, the data obtained by ss-NMR and x-ray diffraction measurements indicated a transformation of the CAM form I to form II after the shortest interval of milling. The gradual disappearance of the typical crystalline peaks in the XRPD spectra was observed with increased milling time, although the time needed for full amorphisation did not correlate with the ss-NMR data. ATR-IR and DSC were the only techniques being able to differentiate between the prepared amorphous samples. In addition the gradual disappearance of signature crystalline peaks can be seen in the AT-IR spectra of CAM samples milled with increased milling times. Differences between the prepared amorphous samples were also seen in the SEM images, though these are of morphological origin, which

does not necessarily indicate a difference in the disorder on the molecular level. The difference in the crystal morphology between CAM form I and II is probably accountable for the difference in time of milling needed for full amorphisation of the forms. To conclude, we have determined that all the utilized methods were suitable to distinguish between the crystalline and amorphous CAM samples. Our results show the potential of such an NMR approach to be used for the assessment of the amorphous fraction of substances in different continuous pharmaceutical processes such as aging, milling and lyophilisation. In addition to that the obtained data shows that, although only detected by AT-IR and DSC, the amorphous samples differ among each other in terms of molecular disorder.

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