Preparation and evaluation of metastable solid-state forms of lopinavir

H.J.R. Lemmer, W. Liebenberg

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H.J.R. Lemmer, Internal Box 36, Private Bag X6001, Potchefstroom, 2520, South Africa
Richard.Lemmer@nwu.ac.za

In this work, we present the preparation and evaluation of previously unreported metastable forms of the antiretroviral drug, lopinavir. By maintaining the chemical structure, physicochemical properties like the glass transition temperature \(T_g\), dissolution and solubility can be readily attributed to the stability of the system. Commercially-available lopinavir was used to prepare partially amorphous crystals, semicrystalline needles, resins and glasses. The physicochemical properties of each were investigated using differential scanning calorimetry (DSC), Fourier transform infrared spectroscopy (FTIR) and powder X-ray diffraction (PXRD). Each sample’s thermal and spectroscopic analyses, as well as dissolution and solubility studies were performed one month after sample preparation, for better comparability. Glass transition temperature, activation energy for global molecular mobility \((\Delta E_{T_g})\), and activation energy for local molecular mobility \((\Delta E_{\text{f}})\) were assessed as primary indicators for structural stability of the systems. Relating these properties to aqueous solubility revealed that each metastable form possessed its own unique equilibrium solubility. Cumulative dissolved fractions \((\alpha)\) were fitted against deceleratory kinetics models, and from the data hereby obtained the dissolution process was determined to followed first-order kinetics \((R^2 = 0.998)\). From the results suggest that multiple metastable solid-state forms of lopinavir can exist under similar conditions, depending on the preparation conditions.

1. Introduction

Lopinavir is a peptidomimetic antiretroviral (ARV) agent which inhibits the human immunodeficiency virus (HIV) protease enzyme from cleaving the Gag-Pol polyprotein, resulting in the production of immature, non-infectious viral particles (Safrin 2004). Its chemical structure is flexible, with 4 chiral centres, and possesses the potential to undergo extensive inter- and intramolecular hydrogen bonding. This structural flexibility enables lopinavir to adopt numerous molecular coordinates on its energy landscape, increasing the probability of preparing various amorphous and crystalline forms (Stillinger 1995). The peptidomimetic structure of lopinavir also imparts an unfavorable pharmacokinetic profile on the drug due to low aqueous solubility, poor absorption and rapid hepatobiliary elimination (Kempf et al. 1997). To address the hepatobiliary elimination, ritonavir is co-administered with lopinavir to outcompete its elimination. The poor solubility of lopinavir can be addressed by preparing amorphous forms of the drug. The high internal energy and specific volume of the amorphous state have already been reported to enhance dissolution, solubility and bioavailability (Hancock and Parks 2000; Hüttenrauch 1978). However, amorphous material is known to be inherently unstable, and will eventually crystallize into a lower energy state.

To date, the glass transition temperature \((T_g)\) and its role with regards to formulation, storage, nucleation and crystal growth, used to be the mainstay parameter for assessing the stability of amorphous systems (Elamin et al. 1994; Levine and Slade 1988; Lordi and Shireman 1984; Mitchell and Down 1984; Ward and Schultz 1995). However, in certain instances the \(T_g\) fails to be an accurate indicator of stability (Vyazovkin and Dranca 2007; Yoshioka et al. 2006, 2007). Recently, local molecular motions \((\beta\text{-relaxations})\) have been recognized as important determinants of the stability of amorphous systems, not only with regards to coupling with global molecular motions \((\alpha\text{-relaxations})\), but also concerning crystallization at temperatures well below and close to \(T_g\), as well as the aggregation of macromolecules (Alie et al. 2004; Alig et al. 1997; Bhugra et al. 2008; Hijima et al. 1999; Ngai 2003; Vyazovkin and Dranca 2005; Vyazovkin and Dranca 2007). An additional advantage of using \(\beta\text{-relaxations}\) to evaluate the stability of amorphous systems, is that it allows investigation into the effects of low temperature and low energy disturbances on the system. The energy barrier imposed by the \(\beta\text{-relaxation}\) can therefore be seen as the first step in re-establishing the energetic equilibrium of molecular mobility in amorphous systems under stress conditions, such as heating.

Studies on amorphous material generally consist of the preparation of an amorphous sample from a fully crystalline reference, either using ball milling, spray-drying, freeze-drying or cooling of the melt, followed by a comparison between the physicochemical properties of the newly formed amorphous sample and the original crystalline material (Crowley and Zografi 2001; Dantuluri et al. 2011; Graeser et al. 2009; Grzybowska et al. 2001;
2010; Hancock et al. 1998; Mahlin et al. 2011; Vyazovkin and Dranca 2006; Weuts et al. 2003). Studies in which more than one method was used to prepare more than one metastable form are sparse (Qi et al. 2008; Surana et al. 2004). Preparation of these amorphous samples either occurs in situ, followed by immediate sample analysis, or the exact amount of time between the sample preparation and the analysis was not reported. It should be noted however, that maintaining storage conditions and time between preparation and analyses were not the objectives of the above mentioned studies. In this study, storage and analysis times were maintained, to explore a different avenue of amorphous stability. Commercial lopinavir was dissolved in various organic solvents under conditions similar to that of a typical recrystallization and cooled under ambient conditions to yield amorphous resins and partially amorphous crystals. Two glasses were also prepared from the melt. One was quench cooled with liquid nitrogen and the other was cooled under ambient conditions to yield glasses with different degrees of disorder (Aasland and McMillan 1994; Ediger 2000; Stillinger 1995). To evaluate the stability of each system, both α- and β-relaxations were investigated. The glasses, recrystallization products and commercial lopinavir were thermally analyzed to determine the ΔEDiss, fragility (m) and strength (D) parameters. These parameters were then correlated with solubility data to investigate their relations. Dissolution data were fitted to deceleratory kinetics models, and from this data the activation energy for dissolution (ΔEact) were obtained. To facilitate comparability, all samples were stored under similar conditions and all analyses were carried out one month after sample preparation.

2. Investigations and results

Recrystallization of lopinavir yielded crystals from acetone and ethyl acetate, semicrystalline needles from diethyl ether and resins from chloroform and dichloromethane. Two glasses were also prepared from the melt. One was quenched with liquid nitrogen and the other was cooled under ambient conditions to yield glasses with different degrees of disorder (Aasland and McMillan 1994; Ediger 2000; Stillinger 1995). To evaluate the stability of each system, both α- and β-relaxations were investigated. The glasses, recrystallization products and commercial lopinavir were thermally analyzed to determine the ΔEDiss, fragility (m) and strength (D) parameters. These parameters were then correlated with solubility data to investigate their relations. Dissolution data were fitted to deceleratory kinetics models, and from this data the activation energy for dissolution (ΔEact) were obtained. To facilitate comparability, all samples were stored under similar conditions and all analyses were carried out one month after sample preparation.

Unfortunately, the exact crystalline content, usually determined using the methods described by Lefort et al. (2004) and Black and Lovering (1977), could not be reliably performed in this in this study, because of the absence of a 100% crystalline sample. However, birefringence could still be used to visualize the crystals present in the more amorphous samples, e.g. the resin and strength (D) parameters of each sample.

Table 1: Values of Tg and Tβ when heated at 10 K/min (mean ± S.D.), activation energies for β- and α-processes and the fragility (m) and strength (D) parameters of each sample

<table>
<thead>
<tr>
<th>Preparation conditions</th>
<th>Tg (K) ± S.D.</th>
<th>Tβ (K) ± S.D.</th>
<th>α -relaxation</th>
<th>β -relaxation</th>
<th>ΔEDiss (KJ/mol)</th>
<th>m</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lopinavir</td>
<td>330 ± 1.56</td>
<td>348 ± 0.41</td>
<td>557</td>
<td>325</td>
<td>144</td>
<td>140</td>
<td>21</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>331 ± 0.23</td>
<td>346 ± 0.67</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Acetone</td>
<td>331 ± 1.24</td>
<td>350 ± 0.06</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>329 ± 0.17</td>
<td>340 ± 2.36</td>
<td>372</td>
<td>251</td>
<td>217</td>
<td>126</td>
<td>33</td>
</tr>
<tr>
<td>Chloroform</td>
<td>329 ± 1.64</td>
<td>339 ± 2.82</td>
<td>250</td>
<td>211</td>
<td>203</td>
<td>165</td>
<td>31</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>329 ± 1.72</td>
<td>331 ± 6.61</td>
<td>368</td>
<td>294</td>
<td>133</td>
<td>94</td>
<td>20</td>
</tr>
<tr>
<td>AG</td>
<td>301 ± 1.04</td>
<td>318 ± 0.08</td>
<td>45</td>
<td>26</td>
<td>302</td>
<td>340</td>
<td>50</td>
</tr>
<tr>
<td>QG</td>
<td>318 ± 0.66</td>
<td>351 ± 0.39</td>
<td>334</td>
<td>273</td>
<td>453</td>
<td>275</td>
<td>67</td>
</tr>
</tbody>
</table>
Fig. 3: Micrograms of lopinavir resin from chloroform (A and B), AG (C) and QG (D), where birefringence was used in B, C and D to visualize the crystallites embedded in the amorphous matrixes

Table 2: Aqueous solubility (mean ± S.D.) of commercial lopinavir, its glasses and recrystallization products

<table>
<thead>
<tr>
<th>Preparation conditions</th>
<th>Solubility (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial lopinavir</td>
<td>54.3 ± 0.7</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>20.4 ± 0.7</td>
</tr>
<tr>
<td>Acetone</td>
<td>24.7 ± 0.6</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>55.3 ± 0.4</td>
</tr>
<tr>
<td>Chloroform</td>
<td>57.9 ± 0.9</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>25.6 ± 0.2</td>
</tr>
<tr>
<td>AG</td>
<td>93.7 ± 0.9</td>
</tr>
<tr>
<td>QG</td>
<td>35.2 ± 0.6</td>
</tr>
</tbody>
</table>

Lopinavir displayed the highest ΔEβ of all the amorphous samples, explaining its stability and resistance to crystallization following shipping, handling and storage for extended periods of time at temperatures below Tg. AG exhibited the highest aqueous solubility (Table 2), followed by the resin obtained from chloroform, while other predominantly amorphous samples, e.g. QG and the resin obtained from dichloromethane, displayed solubility values only slightly higher than those of the crystalline samples. As expected, the predominantly amorphous lopinavir samples displayed enhanced aqueous solubility when compared to their more crystalline counterparts. Based on their physical properties, the crystals from ethyl acetate, QG and AG were chosen for dissolution studies. These samples shared the ability to be broken (without being totally crushed), and served to obtain a similar particle size distribution.
tion, thereby eliminating an important variable that could affect dissolution rate. The gum-like properties of the resins made it impossible for them to be broken down into smaller pieces with a similar size distribution to that of the above mentioned samples. By maintaining the chemical structure, particle size distribution, analysis temperature, stirring rate and dissolution medium, differences in dissolution rate can more readily be attributed to the stability of the system. The dissolved concentrations were expressed as cumulative dissolved fractions ($\alpha$) and straight line regions of the curves (Fig. 5), consisting of $\alpha$ values ranging from 0.1 to 0.8, were fitted against deceleratory kinetics models presented in Table 3. These linear regions represent dissolution under sink conditions. Results from the model fitting indicated that the dissolution process followed first-order kinetics ($R^2 = 0.998$). From the dissolution rate constants, the $\Delta E_{Diss}$ for the lopinavir crystals, QG and AG were determined as 36, 22 and 18 kJ/mol respectively.

### 3. Discussion

As expected, the inherent structural flexibility of lopinavir and its capacity to undergo extensive hydrogen bonding led to a solid-state system presenting multiple metastable forms with varying degrees of disorder, evident from the differences in $T_g$. A physicochemical analysis of lopinavir, its glasses and recrystallization products was undertaken to obtain several parameters which are known to be indicative of differences in the stability of a system, e.g. $\Delta E_T$. During the course of the analyses, several incidents where stability would have been wrongly assigned if only considering $\Delta E_T$ became clear, e.g. commercial lopinavir would have been dubbed the second most unstable lopinavir sample ($\Delta E_T = 144$ kJ/mol). However, it is stable for extended periods of time and is resistant to crystallization following handling, while the resin obtained from chloroform displayed a higher $\Delta E_T$ value of 203 kJ/mol and formed large amounts of crystallites in its amorphous matrix, even while stored well below $T_g$. In both these cases the true physical stability could be deduced from $\Delta E_T$ (Table 1). Despite having a similar macroscopic appearance, the resins from chloroform and dichloromethane displayed vastly different physicochemical...
cal profiles, especially concerning crystalline content. The resin obtained from chloroform had a large amount of crystalline content (Figs. 2 and 3) after one month of storage, while the resin obtained from dichloromethane still displayed only a diffuse halo on its PXRD (not shown). These differences in crystalline content while stored at temperatures below Tg can be attributed to $\Delta E_\beta$, where the lower molecular mobility energy barrier imposed on the resin obtained from chloroform possibly facilitated the formation of crystallites in its amorphous matrix.

Thermal analyses indicated that each solid-state system displayed its own unique $T_g$ and fragility parameters, suggesting that each system is a unique metastable form of lopinavir. This observation was supported by the fact that each sample was analyzed one month after preparation and all samples were stored under similar conditions. Another method of assessing the stability of a system is solubility studies, where unstable systems display higher equilibrium solubility values than their more stable counterparts. By maintaining the chemical structure, physicochemical properties, like solubility, can be readily attributed to the stability of the system. Relating the solubility data to the physicochemical parameters obtained from thermal analyses gave the well known trend in which the solubility increases when moving from crystalline to amorphous systems. However, amongst the amorphous samples the solubility did not increase with increasing amorphous content, as deduced from PXRD (Fig. 4). The best correlation was found between solubility and $\Delta E_\beta$. Interestingly, the $\Delta E_{\alpha,\beta}$ values of the lopinavir samples analyzed exhibited the same trend as their respective $\Delta E_\beta$ values, suggesting that the lower activation energy barrier imposed by lower values of $\Delta E_\beta$ might facilitate the removal of molecules from the solid’s surface, thereby decreasing the amount of energy needed to dissolve the sample and increasing its dissolution rate. However, more future studies will have to be carried out before drawing any conclusions from this potential correlation between $\Delta E_\beta$ and $\Delta E_{\alpha,\beta}$. The solubility and dissolution data support the conclusions drawn from the thermal analyses; that each preparation is a unique metastable form of commercial lopinavir.

In this work, we reported several novel solid-state forms of the peptidomimetic drug lopinavir, and illustrated the ability of a chemical species to rearrange into several metastable solid-state forms, independently existing under similar conditions for up to one month (and possibly even longer). This observation emphasizes the need for pharmaceutical scientists to perform a thorough physical chemical analysis on a drug in order to obtain a true representation of its solid-state properties. Combining several analytical techniques and methods to evaluate the systems, made it clear that each of these metastable forms prepared in this study displayed its own unique physicochemical behavior and stability. The knowledge obtained from this study, e.g. the enhanced aqueous solubility of the lopinavir glass obtained from cooling of the melt under ambient conditions, can assist in future research and formulation of lopinavir and similar peptidomimetic drugs.

4. Experimental

4.1. Materials

Lopinavir (HPLC assay > 99%) was purchased from Dr. Reddy’s Laboratories Ltd., Andhra Pradesh, India.

4.2. Methods

4.2.1. Differential scanning calorimetry (DSC)

DSC experiments were carried out on a Shimadzu DSC-60A (Shimadzu, Japan). The DSC cell was purged with nitrogen at a flow rate of 35 mL/min. Indium and tin standards were used to calibrate the temperature and heat of fusion. All samples were accurately weighed (5–6 mg) and analyzed in Pharmazie 68 (2013) aluminum pans with pared lids to facilitate potential volatile evolution during heating. The data was analyzed using ta60 software version 2.11. To investigate $\beta$-relaxations, the optimum conditions were found to be an annealing time of 20 min at a temperature of 0.8 $T_g$, consistent with known literature (Vvyazovkin and Dranca 2006). Because of the inherently small endothermic peaks associated with $\beta$-relaxations, heating rates of 10, 15 and 20 K/min were used. These heating rates were experimentally chosen based on observations that rates < 10 K/min resulted in extensive oscillation between enthalpy loss and recovery, making accurate data collection troublesome, making 10 K/min the slowest heating rate. Rates $>$ 20 K/min led to overlap of the $\beta$- and $\alpha$-processes, again resulting in unreliable $\beta$-relaxation values and making 20 K/min the highest heating rate used in this study. $T_g$ was taken as the onset temperature of the relaxation, measured using the same extrapolation technique used in $T_g$ determination (Crowley and Zografi 2001). Figure 6 illustrates the small endothermic peaks (relative to the $\alpha$-processes) of the $\beta$-relaxations obtained after annealing, as well as the shifts in onset temperatures with regard to different heating rates. The positions of these peaks, along with their thermal behavior, are consistent with other DSC-based $\beta$-relaxation investigations (Vvyazovkin and Dranca 2006). To investigate $\alpha$-relaxations, the samples were annealed for 10 min at temperatures 20 K above $T_g$ (Crowley and Zografi 2001). The samples were analyzed at heating rates of 5, 10 and 20 K. For both the $\beta$- and $\alpha$-relaxations similar cooling and reheating rates were maintained (Crichton and Moynihan 1988; Morsyehan et al. 1974).

The activation energies for local and global mobility were calculated from non-isothermal DSC data using eq. 1 (Vvyazovkin and Dranca 2006; Crowley and Zografi 2001).

$$ \frac{\Delta E_\beta}{R} = \frac{\Delta H_\beta}{T_\beta} \exp \left( \frac{q}{T_\beta} \right) $$

Where the activation energy ($\Delta E_\beta$) can be written as $\Delta E_\beta$, for local relaxations, and $\Delta E_{\alpha,\beta}$ for global relaxations. From $\Delta E_{\beta}$ the $m$ values were calculated according to eq. 2 (Crowley and Zografi 2001).

$$ m = \frac{\Delta E_\beta}{\Delta T_{\alpha,\beta}} $$

The minimum value of $m$ (m$^{\text{min}}$) was determined to be 16, and from that the $D$ value of each sample was calculated using eq. 3 (Crowley and Zografi 2001).

$$ D = \frac{\text{ln} 10 \Delta H_{\alpha,\beta}^2}{\text{m} - m^{\text{min}}} $$

4.2.2. Thermogravimetric analysis (TGA)

TGA experiments were performed using a Shimadzu DTG-60 (Shimadzu, Japan). The TGA chamber was purged with nitrogen at 35 mL/min. Indium and tin standards were used to calibrate the temperature. All samples were accurately weighed (7–8 mg) and analyzed in open aluminum pans. The data was analyzed using tavo software version 2.11.

4.2.3. Fourier transform infrared spectroscopy (FTIR)

FTIR analyses were performed using a Shimadzu IR Prestige-21 (Shimadzu, Japan). Peak positions were confirmed using polysacrylamid. Spectra were recorded over a range of 500–4000 cm$^{-1}$. All samples were accurately weighed (4–5 mg) and homogeneously dispersed in a ground matrix of KBr.

The data was analyzed using Shimadzu IRR solution software version 1.40.
4.2.4 Ultraviolet-visible absorption spectrophotometry (UV-Vis)

UV-vis analyses were carried out on a Shimadzu UV-1800 spectrophotometer (Shimadzu, Japan). Analyses were conducted at 310 nm in quartz cuvettes using Shimadzu UVProbe software version 3.2.

4.2.5 Hot stage microscopy

Hot stage micrographs were taken on a Nikon Eclipse E400 microscope (Nikon, Japan) equipped with a Nikon DS-F1 camera and cross-polarized light filters. Images were acquired with NIS-Elements software version 3.22.

4.2.6 Powder X-ray diffraction (PXRD)

PXRD analyses were carried out on a PANalytical X’Pert Pro (PANalytical, Netherlands). Measurement conditions were: Cu Kα, λ = 1.5405 Å, 15 K steps/min, step size 0.05°; Generate settings: 40°; Alignment: 0.05°, three frame size; 0.87° in 2θ; scan step times, 19.68°/s, temperature, 25°C. The data was analyzed using X’Pert Data Collector software version 4.0A.

4.2.7 Solubility

Aqueous solubility of each sample was determined by stirring a supersaturated solution of each prepared material at 310 K for 24 h to reach equilibrium solubility. Concentrations were determined by measuring the UV-vis absorbance. Linearity was observed in the range 0.01 to 200 μg/mL.

4.2.8 Dissolution

To control the particle size distribution, samples were sieved and the fraction between 400 and 1400 μm collected. Accurately weighed (54–56 mg) amounts of these samples were dissolved in 10 mL, double distilled water at 298, 308 and 318 K over 4 h. Initial withdrawals were made at 1 minute intervals for the first 5 min, followed by longer time intervals and analyses were carried out using UV-vis absorbance.

Normalized cumulative dissolved fractions (αt) were fitted against decimal logarithms on a computer using the well-known Arrhenius equation (Eq. 4).

\[ \alpha_t = A e^{- \frac{k}{T}} \]

Where \( \alpha_t \) is the fraction dissolved and \( T \) the gas constant.

References


mine glass transition temperature and molecular mobility below \( T_g \) for amorphous drugs. Int J Pharm 259: 17–22.
