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RESEARCH ARTICLE

Preparation and characterization of polymorphs of the glucocorticoid deflazacort

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Abstract

The polymorphism of new and old active pharmaceutical ingredients (APIs) is of great importance due to performance, stability and processability aspects. The objective of this study was to investigate the polymorphism of deflazacort (DEF), a glucocorticoid discovered >40 years ago, since this phenomenon has not been previously investigated for this API. Using different methods for solid form screening, it was determined for the first time that DEF is able to exist as three forms: a crystalline (DEF-1); a hydrated X-ray amorphous (DEF-t-bw) and an anhydrous amorphous phase (DEF-g) obtained from manually grinding DEF-1. The *in vitro* and *in vivo* dissolution rates (DRs) of DEF-1 and DEF-t-bw, which were measured using the rotating disk method in water at 37 °C and the pellet implantation technique in rats, respectively, indicated that DEF-t-bw exhibited slightly faster *in vitro* and *in vivo* DRs than those of the crystalline form, but the values were not significantly different. In addition, it was determined that DEF-t-bw devitrifies to DEF-1 by the effect of pressure, humidity and heat. It was concluded that DEF is glucorticoid with low tendency to exhibit different crystalline forms and that DEF-t-bw has no advantages over DEF-1 in terms of solubility, DRs and solid-state stability.

Keywords

Crystallization, deflazacort, disk intrinsic/*in vivo* dissolution rates, polymorphism, powder X-ray diffractometry

History

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Introduction

Pharmaceutical active ingredients (APIs) are able to exist as true polymorphs (crystalline forms with different arrangements and/or conformations of the molecules in the crystal lattice), amorphous forms and solvates, which is a behavior known as polymorphism[1–3]. Undoubtedly, different solid-state forms will differ in terms of physical, mechanical and chemical properties[4]. These properties can have a direct effect on the ability to process and/or manufacture the drug substance and the drug product, as well as on drug product stability, dissolution and bioavailability[2]. Thus, polymorphism can affect the quality, safety and efficacy of the drug product[2].

The fact that APIs may exist in a multitude of physical forms allow pharmaceutical scientists to select the most suitable form to be included in a product formulation[4]. Although the most common approach in solid dosage form development remains using the thermodynamically stable crystalline state of an API to be formulated, a promising alternative lies in the transformation of the crystalline drug into its amorphous state[5]. The amorphous form of an API has frequently been shown to have a higher solubility and therefore often a higher bioavailability. Compared to the crystalline state, the amorphous form of an API has excess entropy, enthalpy and free energy that accounts for its improved solubility[5]. However, because of the excess thermodynamic properties, the amorphous state is inherently unstable and recrystallization may occur[5], which generates lack of confidence in the behavior of amorphous APIs. Important legal factors related to patent issues are also involved, since true polymorphs and amorphous forms can be patented separately if they have some advantages compared to the phase which is in use[6]. For these reasons, new and old APIs have been screened to search for the existence of multiple crystalline or amorphous phases[7–9].

Deflazacort, 1-(1β,16α)-21-(acetyloxy)-11-hydroxy-2′-methyl-5′H-pregna-1,4-dieno [17,16-*d*] oxazole-3,20-dione (DEF, Figure 1), is a glucocorticoid used systemically in Europe, India and South America for the treatment of various disease states such as allergic reactions, asthma and rheumatoid arthritis, and especially in the prevention of rejection in transplanted organs[10,11]. DEF is a poorly water-soluble API that was patented in 1966[12], and is usually formulated as tablets. Although efforts have been directed toward understanding the *in vivo* behavior of DEF[13,14] little is known about its polymorphic behavior, despite polymorphism being a very common phenomenon in the steroid family[8,9,15].

Recently, we have resolved the crystalline structure of DEF and studied its spectroscopic and thermal properties[16]. The obtained results demonstrated that DEF crystallizes from ethyl acetate in the orthorhombic system, space group $P2_12_12_1$, with $Z=4$ and a crystal structure being stabilized by intra- and intermolecular hydrogen bonds, which led to a very closely packed form, hereafter named DEF-1.

In view of the importance of polymorphism in steroids, the aim of this study was to investigate the possible existence of different

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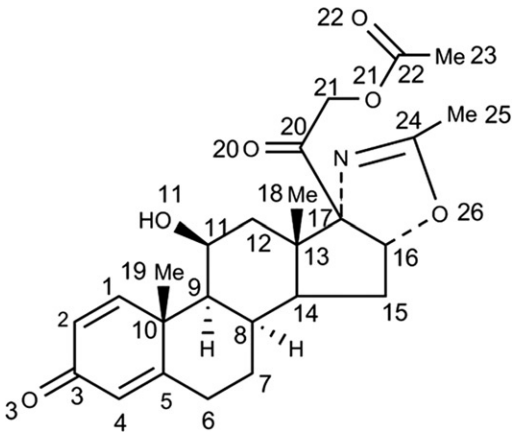

Figure 1. Chemical structure of deflazacort (DEF) and atomic numbering used in this study.

solid forms of DEF. Several solid samples of DEF were obtained using different organic solvents and crystallization techniques, which also included crystallization with polymer heteronuclei[17] and lyophilization. The isolated samples were characterized by means of X-ray powder diffractometry (XRPD), thermal analysis (differential scanning calorimetry and thermogravimetry), hot stage and optical microscopy (HSM and OM) and diffuse reflectance infrared (DRIFT). The intrinsic dissolution rates (DRs) of the samples that represented the different physical phases were analyzed based on the rotating disk method. *In vivo* studies were also performed using the implantation technique in rats described by Haleblian et al.[18].

Materials and methods

Materials

Deflazacort raw powder (DEF-rp, micronized sample, 99.2% purity) was kindly provided by Sanofi-Synthelabo of Argentina. Spectral grade Potassium bromide (KBr) was purchased from Merck, Argentina. All the solvents used were of analytical or HPLC reagent grade and Milli-Q water (Millipore®, Bedford, MA) was utilized. For thin layer chromatography (TLC) analyses, dichloromethane/methanol (9:1) and pre-coated plates of silica gel 60 F254 (Merck Chemicals) were used as the eluting solvent system and stationary phase, respectively. Spots were visualized with UV light and iodide vapors. Nylon membranes (0.45 μm pore size, Pall Corporation, Port Washington, NY) were commercially acquired.

Preparation of DEF samples

There are a large number of classical and innovative techniques that can be used to generate different polymorphs of an API. In this study, the polymorph screening were conducted by crystallization from solution (by cooling a supersaturated solution, evaporating solvent and adding a poor solvent to a solution), lyophilization, grinding and polymer induced heteronucleation. For the crystallization methods, the solvents were selected taken into account various properties (i.e. polarity, hydrogen bonding propensity and solubility/insolubility of DEF) as the solvent is one of the secondary factors affecting the crystallization outcome, mainly through their effect on the degree of supersaturation[19]. The solvents used were acetone (ace), benzene (ben), carbon tetrachloride (CCL_4), chloroform (Chlor), dioxane (diox), ethanol (EtOH), ethyl acetate (ea), hexane (hex), isopropanol (isoOH), methanol (MeOH), *t*-butanol (t-b) and water. The polymorph screening procedures were the following: (Method A) *Recrystallization:* Saturated solutions were prepared in boiling solvents and rapidly filtered (Whatman 532 paper) into open vials. The vials were covered and allowed to cool slowly to 20–25 °C (room temperature, RT). (Method B) *Slow evaporation*: Saturated solutions were prepared at RT and filtered (Whatman 532 paper) into open vials. The vials were covered with filter paper and allowed to evaporate at RT. (Method C) *Fast evaporation*: Saturated solutions were prepared at RT. After filtration, the solutions were evaporated in a rotavapor at 35 °C. (Method D) *Fast cooling*: Saturated solutions were prepared in boiling solvents and rapidly filtered (Whatman 532 paper) into open vials. The vials were cooled at −20 °C and kept in a freezer to allow crystallization. (Method E) *Precipitation with an antisolvent:* Portions of DEF-rp were dissolved in boiling ea, and hex or Cl_4C were added up to the apparition of a persistent precipitate. (Method F) *Lyophilization*: A solution of DEF (30 mg/mL) in t-b-water 4:1 was frozen with liquid air and lyophilized at −40 °C for 24 h (Freezone 6 Freeze Dry System, Labconco®, Kansas City, MO). Then, the resultant solid (named DEF-t-bw) was subjected to a secondary drying in vacuum (RT, P_2O_4) for 24 h. (Method G) *Grinding*: An aliquot was manually ground with mortar and pestle for 45 min. (Method H) *Heterogeneous nucleation*: The method of Price et al.[17], which consists of the use of insoluble polymers as heteronuclei to search for organic polymorphs, was applied. To select the polymers, pieces of polymers (3–10 mg) were placed in screw-capped vials and 10 mL of MEOH was added. After 48 h, the polymers were examined visually and the supernatants subjected to TLC analyses. Polymers selected were those that did not swell or solubilize in MEOH, which were hydroxypropyl methylcellulose (HPMC), pellethane, polypropylene, high-density polyethylene (HDPE), polystyrene, PVC film and three investigational polymers (M_4, M_5 and M_7). To crystallize DEF, aliquots (0.45 mL) of a methanolic solution (20 mg/mL) were added to the polymer-containing microtiter plates such that 9 mg of DEF-rp was dispensed into each well. The plates were covered with filter paper, and the solvent was allowed to evaporate at RT. Solids that formed were isolated and examined by HSM. Studies were performed in duplicate. Due to the photoinstability of DEF[16,20], all the solutions as well as the solid samples were protected from the light.

General methods

X-ray powder diffractometry

X-ray powder diffractometry patterns were collected at RT on a Bruker AXS D8 ADVANCE X-ray powder diffractometer [Bruker, Germany, fitted with a Copper tube (Cu $K\alpha =$ 1.54178 Å) and a post-diffraction graphite monochromator] and/or a PANalytical X′PERT PRO diffractometer [Philips, Netherlands, fitted with a Copper tube (Cu $K\alpha = 1.54178$ Å) and a Ni filter]. In both cases, the X-ray generator was set at a voltage of 40 kV and current of 30 mA. XRPD scans were recorded in step mode with a step size of 0.05° 2θ and step time of 3 s over an angular range of 3–30° 2θ. A 25-mm diameter Si single crystal holder was used. The sample holder was rotated in a plane parallel to its surface at a speed of 30 rpm during the measurement taking (Bruker AXS D8 ADVANCE diffractometer). Samples gently grinded were pressed by means of a clean glass slide to ensure coplanarity of the powder surface with the surface of the holder. Diffractograms were analyzed with X′PERT Data Viewer diffraction software.

Thermo- and stereomicroscopy

The physical and morphological changes in the samples that occurred during heating were observed through a microscope fitted with a Kofler hot-stage (Leitz, Wetzlar, Germany) at

a constant rate from RT (at ~8 °C/min) up to 265 °C. The shape and surface characteristics of solid samples were also examined under a stereomicroscope (StereoZoom® Leica S8 APO with apochromatic 8:1 zoom, Leica Microsystems, Wetzlar, Germany) with an adapted digital camera system.

Differential scanning calorimetry and thermogravimetry

Differential scanning calorimetry (DSC) and thermogravimetry (TG) measurements were recorded on MDSC 2920 and TG 2950 analyzers (TA Instruments Inc., New Castle, DE), respectively. Accurately weighed samples (1–2 mg) were analyzed in crimped and/or hermetic aluminum pans. Different heating rates (10 and 20 °C/min), under a nitrogen (99.99%) purge of 50 mL/min, were applied. The DSC and TG temperature axes were calibrated with indium (99.999%, m.p. 156.60 °C) and the Curie point of Ni (358.14 °C), respectively. Empty aluminium pans were used as references. The reported DSC values were the average of at least two independent measurements. Data were treated with Universal Analysis 2000 software (TA Instruments Inc.).

Diffuse reflectance infrared Fourier transform

Diffuse reflectance infrared Fourier transform (DRIFT) spectra were recorded on a Nicolet Avatar 360 spectrophotomer (Nicolet Instruments Corp, Madison, WI). A diffuse reflectance accessory and macro diffuse reflectance cups of 13 mm diameter (~400 mg) were used. For the preparation of the blend, dry KBr was lightly ground with an agate mortar and pestle for 2 min before being mixed with the sample (2.5% w/w). The blend was then placed in the cup, and excess material was removed by placing a microscope slide against the open cup in a rotary motion, to leave a level but roughened surface, and scanned immediately. DRIFT spectra were acquired accumulating 64 scans at a 4 cm^{-1} resolution. All spectra were processed with the OMNIC E.S.P. 5.1 program (Nicolet Corp.). KBr scans were used as background.

Aqueous solubility and intrinsic DR measurements

For solubility measurements, excess amounts (~10 mg) of a sample crystallized from ethyl acetate by the Method A (named DEF-ea and taken as representative of the anhydrous crystalline form of DEF, i.e. DEF-1) and DEF-t-bw were introduced into screw-capped glass vials containing 7 mL of Milli-Q® water and the tubes were stoppered and placed in a shaking water bath maintained at 37 °C. Since preliminary studies have indicated that DEF-ea and DEF-t-bw are stable in Milli-Q® water at 37 °C up to 6 h of testing, two aliquots of the solutions were withdrawn with a syringe at 6 h, filtered (0.45 μm membrane) and analyzed by RP-HPLC (isocratic mode; MEOH:water [80:20 V/V], Synergi-4μ-Fusion RP-80 C_{18} [Phenomenex, 250 mm × 4.6 mm, 4.5 μm] column and UV-visible detection at 254 nm)[20]. Studies were performed in duplicate.

Disk intrinsic DR (DIDR, ***J***) were measured with a USP rotating disk apparatus[21] (surface area: 0.5 cm^2) on a Hanson SR6 dissolution tester (Hanson Research, Chatsworth, CA) using 200 mL of deaerated Milli Q water at 37.0 ± 0.5 °C and 50 rpm (the recommended speed to maintain reproducible laminar flow of the dissolution medium[22]). Before conducting the DIDR studies, the compression force to obtain non-disintegrating disks was selected and the drug powder (100 mg) was compressed at compactation forces ranging from 100 to 800 kg (1102–8820 psi) for 1 min. Below 600 kg (6610 psi), the powder was not well compacted whereas above this compression force devitrification of DEF-t-bw to DEF-1 was noted by XRPD. No polymorphic conversions were revealed for DEF-ea at any of the assayed pressures. A compromised compression force of 600 kg (6610 psi) was therefore found to produce a disk with acceptable mechanical properties. For all experiments ($n = 3$), aliquots (3 mL) were withdrawn (with replacement) at time intervals of 30, 40, 50, 60, 90 and 120 min, filtered (0.45 μm membrane) and analyzed by HPLC[20]. In all cases, the first milliliter was discarded. For quantification of DEF, a standard curve was prepared using six concentration levels. All the sample solutions were protected from the light until being analyzed. The cumulative amount of dissolved drug per unit area (μg/cm^2) was plotted against time, and linear regression of the data was performed. The value of ***J*** of the test specimen was determined by the slope of the regression line[21,23]. After the dissolution experiments, the dies were examined under the stereomicroscope and no eroded edges, holes or lines on the surface of the pellets were detected. XRPD analyses of the remaining disks revealed that both samples corresponded to DEF-1 thereby indicating the total devitrification of DEF-t-bw.

In vivo *DR studies*

The *in vivo* DRs *were* determined by the pellet implantation technique described by Haleblian et al.[18]. Accordingly, thin, cylindrical pellets of DEF-ea and DEF-t-bw (7mm in diameter), having a mean weight of ~50 mg, were compressed (Hidraulicos Delfabro press, Argentina) at 600 kg for 15 s. Six adult male Wistar rats (mean weight of 185 g) were used to test each solid phase. Six additional animals were used as sham-operated controls and another six animals as non-operated controls. Each animal of each test group (DEF-ea or DEF-t-bw) was implanted subcutaneously in the abdominal area with two pellets of the same test sample. The animals were anesthetized with a ketamine (55 mg/kg)–xilacine (11 mg/kg) mixture, and a ventral midline incision was made. The subcutaneous connective tissue lateral to the incision was teased apart, and the pellets were implanted with the incision being closed by suturing after implantation. The subcutaneous connective tissue lateral to the incision was teased apart, and the pellets were implanted with the incision being closed by suturing after implantation. The animals were allowed food and water *ad libitum*. After 72 h, the animals were sacrificed, the pellets were removed, cleaned of extraneous organic material, dried, weighed and their average surface area determined using a gauge. The mean *in vivo* DR per surface area of pellet (DR_{IV}) was calculated for each phase according to:

$$DR_{IV} = \frac{\Delta W}{\Delta t . \Delta A} \quad (1)$$

where ΔW is the weight change of the disks; Δt is equal to 72 h (the animal time exposition to the implanted pellets) and ΔA is the pellet area variation[18]. The animal weight loss was also determined to complement the DR_{IV} results. The animals were weighed daily, and the percentage loss in original body weight was calculated. All animal experiments were performed according to an approved protocol (Resolution 45/2010 of Animal Ethics Committee of Facultad de Ciencias Químicas, UNC).

Solid-state stability

To assess thermal/moisture solid-state stability of DEF-t-bw and DEF-ea, 200 mg of each solid form were stored in screw-capped glass vials at −20 °C for 365 days and at 25 °C for 3 months. Both samples were also placed in open glass vials at 40 °C/75% RH in a humidity stability chamber. TLC, XRPD and TG were used to evaluate chemical purity, devitrification or polymorphic conversions of the samples after storage at the assayed conditions.

Table 1. Methods of preparation, m.p. (°C) and crystalline habits of DEF samples.

Method[a]	Solvent[b]/polymer	m.p. (°C)[c]	Habit
A	ea/–	256	Prismatic + acicular
A	ace/–	257	Prismatic + acicular
A	diox-w (1:2,5)/–	257	Prismatic
A	ETOH/–	257	Prismatic + acicular
A	isoOH-water (2:1)/–	257	Prismatic + acicular
A	MEOH/–	257	Prismatic + acicular
A	MEOH-water (3:1)/–	257	Prismatic
A	t-b/–	257	Laminar
B	ea/–	256	Prismatic
B	ben/–	256	Laminar
B	chlor/–	256	Prismatic
C	chlor/–	256	No[d]
D	ea/–	257	Acicular
D	ETOH/–	257	Acicular
D	isoOH/–	257	Prismatic
D	isoOH-water (2:1)/–	256	Prismatic + acicular
D	MEOH/–	257	Acicular
E	ea + hex/–	257	Acicular
E	ea + CCl_4/–	256	Acicular
F	t-b-water (4:1)/–	257	No[e]
G	–	257	No[d]
H	MEOH/HPMC	256	Prismatic
H	MEOH/pellethane	257	Laminar
H	MEOH/M_7	256	Acicular
H	MEOH/M_5	256	Acicular
H	MEOH/M_4	257	Laminar
H	MEOH/polypropylene	257	Laminar
H	MEOH/HDPE	257	Acicular
H	MEOH/polystyrene	257	Acicular
H	MEOH/PVC (film)	257	Prismatic
H	MEOH/chitosan	257	Acicular
DEF-rp	–	255	No[d]

[a]A: recrystallization; B: slow evaporation; C: fast evaporation; D: fast cooling; E: antisolvent addition; F: lyophilization; G: grinding; H: polymer heteronucleation. [b]ace: acetone; ben: benzene; chlor: chloroform; dioxane: diox; ea: ethyl acetate; ETOH: ethanol; isoOH: isopropanol; hex: hexane; MEOH: methanol; t-b: *tert*-butanol; t-b-water: *tert*-butanol-water. Polymers: HPMC: Hydroxypropylmethylcellulose and HDPE: High-density polyethylene. [c]Determined by thermomicroscopy (accuracy ± 2 °C). [d]Cryptocrystalline solid. [e]Anhedral solid.

Results and discussion

Melting points and morphological features of prepared samples

To investigate the polymorphism of DEF, 30 solid samples were prepared, with all of these found to be chromatographically pure according to the TLC results. The resultant solids were initially examined by thermo- and stereomicroscopy for melting behavior and morphology and Table 1 summarizes the melting points (m.p.) and the morphological features of the isolated samples. All the determined m.p. were similar (256–257 °C), suggesting that the samples were isomorphic. In addition, when the solid particles were embedded in silicone oil, and heated to render the possible existence of solvates visible, no evaporation losses were detected for any sample, except for the one obtained by lyophilization (DEF-t-bw). This result ruled out the formation of solvates, with the exception of DEF-t-bw, where a few tiny bubbles were observed at ~95 °C indicating the loss of solvent from the material. Regarding the morphological features, the samples were euhedral solids, except those obtained by lyophilization (DEF-t-bw) and grinding (DEF-g), which were anhedral solids, and that of chloroform (DEF-chlor), which was a cryptocrystalline solid like DEF-rp. The predominant crystal habits were the prismatic and the acicular, with particles being colorless or pale-yellow (Supplementary Material 1).

XRPD, DSC, TG and DRIFT

X-ray powder diffractometry patterns of DEF samples, except for DEF-t-bw and DEF-g (whose diffractogram could not be obtained due to the grinding introducing static electricity onto the powder thus making it very cohesive), showed sharp diffraction peaks, indicating that all were crystalline materials. Figure 2 exhibits the diffractograms of representative samples. As shown in Figure 2, the powder patterns of the samples crystallized from ethyl acetate (DEF-ea, Figure 2b), benzene (DEF-ben, Figure 2c), chloroform (DEF-chlor, Figure 2d) and high density poly-ethylene (DEF-HDPE, Figure 2e) were coincident with that of DEF-rp (Figure 2a), indicating that they corresponded to the same crystalline phase, i.e. DEF-1[16]. Differences in peak intensities were observed, but they can be attributed to preferred orientation that could not be eliminated in sample preparation. Subsequent to the above analysis, we were alerted to a recent report on the preparation of hollow crystals of DEF by antisolvent crystallization from methanol-water[24]. According to Paulino et al.[24], the prepared sample exhibited the same crystalline structure that two commercial raw powders of the Brazilian market, which were used as references. Interestingly, the diffractograms of our crystallized samples (Figure 2b–e) were in accordance with the one reported by Paulino et al.[24], indicating that our samples exhibited the same crystalline structure that their samples (i.e. DEF-1), and this provided an additional support to our observation that by different crystallization methods neither solvates nor different polymorphs are obtained. In contrast, the difractogram of DEF-t-bw (Figure 2f) was devoid of diffraction peaks and a halo pattern was observed, revealing the lack of three-dimensional long-range molecular order.

The behaviors on heating of DEF-t-bw and DEF-g were assessed and compared with those of DEF-rp and DEF-ea (taken as representative of DEF-1). The DSC and TG curves of DEF-rp and DEF-ea (Figure 3a and b) matched well, indicating that they had no thermal differences and were solvent-free solids, as neither DSC desolvation nor TG weight losses were observed <220.0 °C. The DSC scans exhibited single melting peaks with extrapolated onsets (T_{onset}) at 257.2 °C and 257.5 °C, respectively, and with decomposition effects occurring >300.0 °C. It should be noted that the DSC profiles of the rest of the crystalline samples (figures not shown) were similar to those of DEF-rp and DEF-ea. In contrast, the DSC curves of DEF-t-bw and DEF-g showed differences with DEF-rp and DEF-ea in the thermal effects before melting. The DSC scan of DEF-t-bw (Figure 3c) displayed three thermal events: a small broad endotherm picking at 68.2 °C that was superimposed with a broad exothermic peak at 98.3 °C (T_{peak}), resembling a glass transition overlapped with a crystallization event[25–27], and an endothermic peak at 254.8 °C (Tonset) due to melting of the compound. According to the respective TG curve, the endotherm at 68.2 °C was related to an average weight loss of 4.2%. Therefore, it cannot be only attributed to a glass transition since a desolvatation phenomenon also occurred. Considering that DEF-t-bw was obtained by lyophilization from t-b-water and that both solvents can remain in lyophilized solids[28], the sample was assessed by Karl-Fisher titrimetry [Metrohm 702 SM Titrino (Herisau, Switzerland), calibrated with deionised water before sample analysis]. The water content found was 4.5% w/w, a value similar to the weight loss determined by TG (4.15%) and thus the presence of residual *t*-butanol was ruled out. The DSC curve of DEF-t-bw was also obtained at 20 °C/min (curve not shown) because glass transition and desolvation are sensitive to scan rates[27,29]. As expected, the endo- and exothermic peaks enlarged and shifted to higher temperatures, thereby confirming that both peaks represented

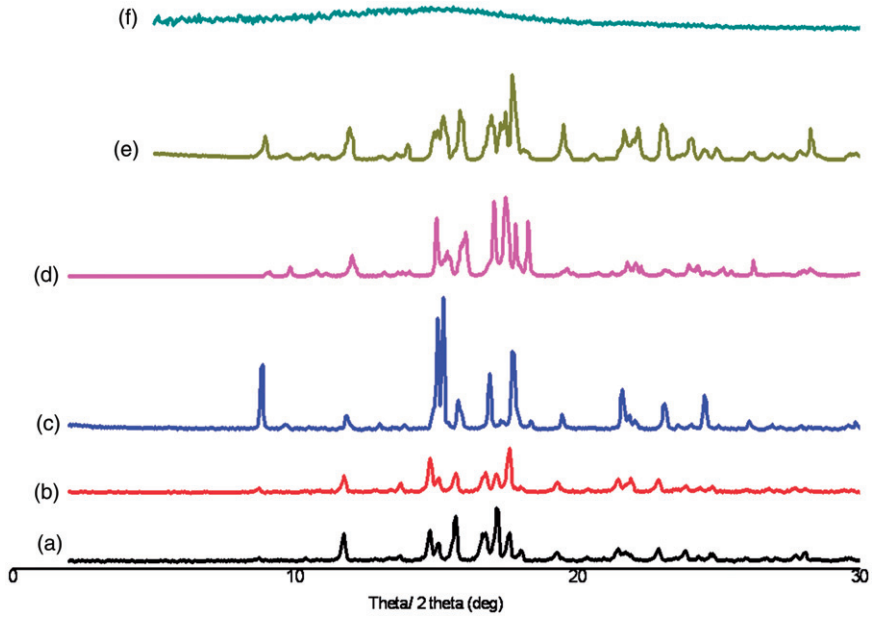

Figure 2. XRPD patterns of selected DEF samples. Key: (a) DEF-rp, (b) DEF-ea (Method A), (c) DEF-ben, (d) DEF-chlor, (e) DEF-HDPE and (f) DEF-t-bw.

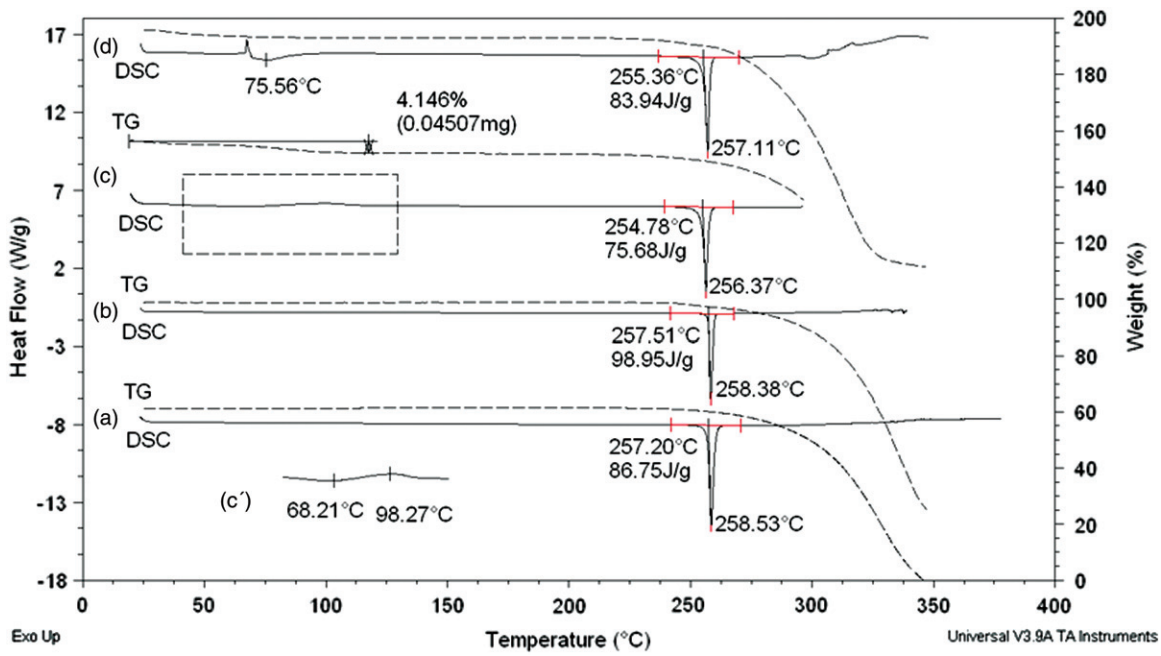

Figure 3. DSC (crimped pan) and TG curves of selected DEF samples (10 °C/min, flowing N_2 at 50 mL/min). (a) DEF-rp, (b) DEF-ea (Method A), (c) DEF-t-bw, (c′) Inset: DSC curve of DEF-t-bw in the 40–140 °C temperature range and (d) DEF-g.

kinetically controlled events[27]; however, this experimental condition was not appropriate to resolve these peaks. Thus, the DSC curve of DEF-t-bw may be interpreted as follows: the X-ray amorphous sample dehydrated and crystallization of DEF occurred. Most likely, the glass transition was overlapped with the desorption endotherm since a crystallization event was detected by DSC (Figure 3c), indicating the devitrification of an amorphous phase[27,30]. Further studies using other analytical techniques are necessary to determine the glass transition temperature of DEF-t-bw.

The DSC curve of DEF-g (Figure 3d) exhibited a broad small endothermic peak at 75.6 °C (T_{peak}), which was preceded by a small exothermic event, and a melting peak at 255.4 °C (T_{onset}). According to the TG curve (Figure 3d), the DSC events between 50 °C and 100 °C were not related to weight losses, and therefore the peak at 75.6 did not represent a desorption process. The initial DSC exotherm could represent the rearrangement of the defective regions to a configuration closer to the original crystal or the crystallization of disordered molecules of the surface of the amorphous particles[31] since grinding causes a dramatic disruption of the local arrangement of the molecules, which may include the formation of new surface, cracks, defects, higher energy molecular conformations, nuclei and so on[31]. The endotherm picking at 75.6 °C could be indicative of a glass transition event of the bulk of the particles[31] that is reflected in a DSC curve by a baseline jump or a broad endothermic peak[31–33]. Unfortunately, the diffractogram of DEF-g could not be obtained (due to its high cohesivity), and therefore it could not be established that the sample was X-ray amorphous. However, using polarizing light microscopy (PLM), it was determined that DEF-g showed no

birefringence (Supplementary Material 2), indicating that it was an isotropic material. Thus, the DSC, TG and PLM evidence indicate that grinding of the crystalline form of DEF resulted in the formation of an anhydrous amorphous form.

In order to obtain information about intra- and intermolecular interactions in DEF-t-bw, its DRIFT spectrum was compared with that of DEF-ea. As shown in Figure 4, the spectra showed differences, with that of DEF-t-bw exhibiting a generalized broadening and diffusion of bands, typical of amorphous phases that had broader distribution of bond lengths and energies with respect to the crystalline counterparts[34]. For example, DEF-ea showed the stretch vibration of the 11-OH as a broad band at 3453.9 cm^{-1}, which is the typical range for a hydroxyl group associated through H-bonds, while in DEF-t-bw, the band was broader and was centered at ~3447.9 cm^{-1}. The downshifting and broadening of the OH band might result from a stronger association of the OH group in DEF-t-bw than in DEF-ea and the presence of water molecules, as indicated by KF titration. The absorptions due to the carbonyl groups (22-CO, 20-CO and the 3-CO) also displayed differences. In DEF-ea, these bands appeared at 1747.7, 1729.2 and 1651.0 cm^{-1}, respectively, whereas in DEF-t-bw although the 22-CO and the 3-CO bands shifted upward (1751.0 and 1658.3 cm^{-1}), the 3-CO stretch remained unchanged, signifying the absence of any interaction involving the cyclic α–β unsaturated 3-CO. The C–H stretching vibrations corresponding to saturated and unsaturated carbons, and the symmetric and anti-symmetric stretching of the CH_2 and CH_3 groups also exhibited differences in both samples. In DEF-ea, the vibrations appeared as several sharp bands at around ~2920 cm^{-1}, while in DEF-t-bw the bands were diffused, suggesting increased interactions involving the CH_2 and CH_3 groups upon randomization of the molecular arrangement.

Aqueous solubility and intrinsic DR measurements

The apparent aqueous solubility DEF-t-bw and DEF-ea was measured to determine if the former presented solubility advantages over DEF-ea. The concentration values at 6 h, which were free of degradation products were taken to be a measure of the saturation concentrations[20,35] and were found to be 108 μg/mL (DEF-ea) and 157 μg/mL (DEF-t-bw), respectively. This indicated that the amount dissolved from DEF-t-bw was ~37% higher than that from DEF-ea, which led to an apparent solubility ratio of 1.44 and a less than 0.5-fold solubility advantage. Several reports in the literature indicate that the solubility advantage of amorphous forms may be quite significant for example, 1.4-fold for indomethacin, 2.5-fold for tetracycline, and ~10-fold for novobiocin acid[36]. Although the estimated solubility ratio for DEF-t-bw and DEF-ea is probably underestimated due to chemical instability of DEF, the obtained solubility ratio suggested that the solubility advantage of DEF-t-bw is not significant to be translated into a biopharmaceutical advantage.

Disk intrinsic DR studies were also performed as $\boldsymbol{J}$ is proportional to the solubility of different polymorphs[37] and will be a true reflection of initial rate of solubility for amorphous and crystalline forms of APIs. The mean DIDR profiles for both phases are shown in Figure 5. As evident from the figure, there is no appreciable difference in the profiles of DEF-ea and DEF-t-bw, which produced linear curves with correlation coefficients of 0.9992 (DEF-ea) and 0.9997 (DEF-t-bw). The calculated values of $\boldsymbol{J}$ were 9.20 and 7.76 μg min^{-1} cm^{-2} for DEF-t-bw and DEF-ea, respectively, where the former is having a slightly higher $\boldsymbol{J}$ but the values were not statistically different ($p = 0.31$, statistical *t*-Student's test at the 0.05 significance level), which may be explaining by considering that DEF-t-bw devitrified by effect of the compactation force and the dissolution medium (Experimental section). Thus, DIRD studies also indicated that DEF-t-bw has no DR advantages.

In vivo *DR studies*

The calculated *in vivo* DRs (DR_{IV}) of DEF-t-bw and DEF-ea compacts were 0.12 (DEF-ea) and 0.16 mg/h/cm^{-2} (DEF-t-bw), but the values were found not significantly different ($p = 0.07$, statistical *t*-Student's test at the 0.05 significance level), and this results are in accordance with the DIDR data which indicated that there is no DR advantages for DEF-t-bw. In addition, the animal weight loss was measured as a parameter related to

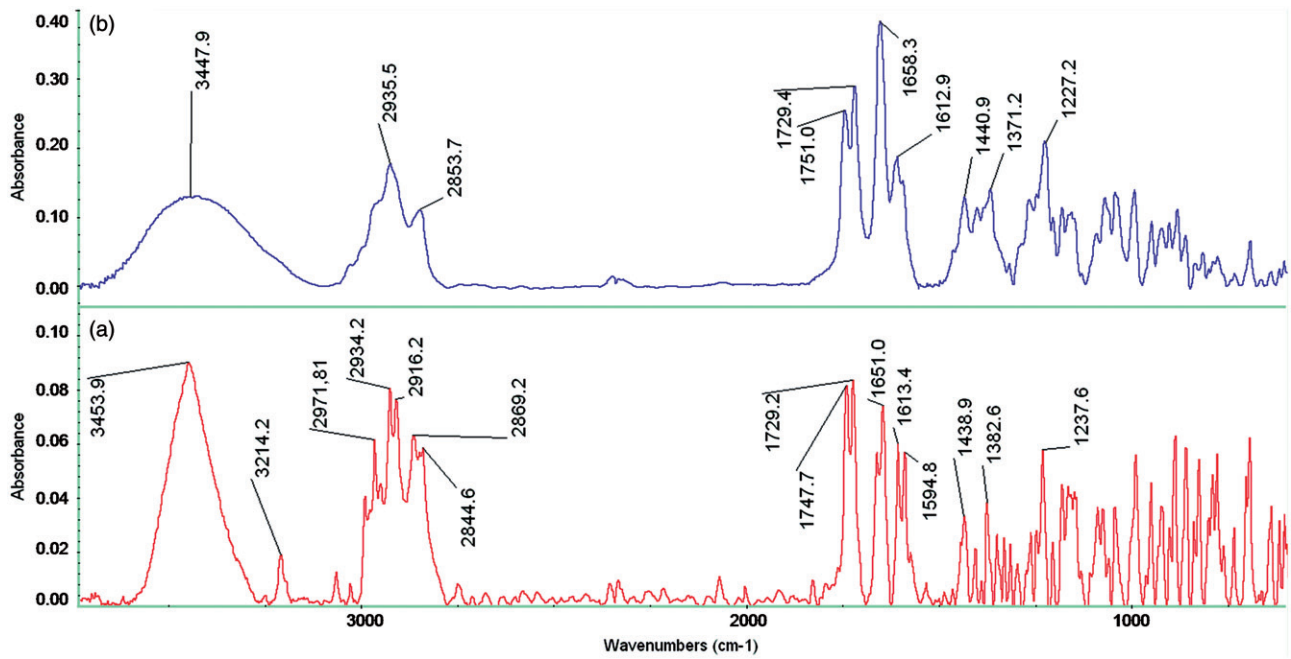

Figure 4. DRIFT spectra of (a) DEF-ea (Method A) and (b) DEF-t-bw.

the pharmacological effect of the API and Figure 6 shows the variations in the animal weights for the control and the implanted groups of rats. Both control groups enhanced their weight (Figure 6a and b) because the animals kept feeding as usual. However, in the case of the implanted groups, a loss weight was detected (Figure 6c and d). The animals treated with DEF-t-bw showed a slightly greater weight loss than those with DEF-ea, but there was also no significant differences in the weight losses.

Solid-state stability

The effect of temperature/humidity on the devitrification and chemical stability of DEF-t-bw was studied under three conditions (−20 °C/0% RH, 25 °C/0% RH and 40 °C/75% RH) in comparison with DEF-ea. DEF-t-bw did not show any signs of recrystallization when stored at −20 °C and 0% RH for 1 year. Indeed, the diffractograms obtained at different times were similar and showed no differences with the initial one, i.e. the amorphous halo was centered in all the traces at ∼15° 2θ (Figure 7a). In addition, the color and appearance of the sample did not change, and by TLC analyses no degradation products were detected, indicating that DEF-t-bw was chemically and physically stable in this condition for 1 year. No devitrification and degradation were found when DEF-t-bw was stored at 25 °C/0% RH up to 3 months; however, signs of devitrification were noted when tested after 1 year of storage at this condition (Figure 7b). Exposure of DEF-t-bw to 40 °C/75% RH resulted in partial reversion into its crystalline form when tested after 7 days (Figure 7c), but no chemical degradation was observed by TLC. Nevertheless, after 30 days of storage, the color and appearance of the sample changed, and degradation products were detected by TLC, indicating that temperature and humidity promoted the devitrification and degradation of DEF-t-bw. In contrast, DEF-ea was physically and chemically stable in the three assayed conditions.

Conclusion

This study contributes to currently knowledge about DEF by investigating some unexplored aspects of its solid state properties. It was determined for the first time that DEF exists in at least

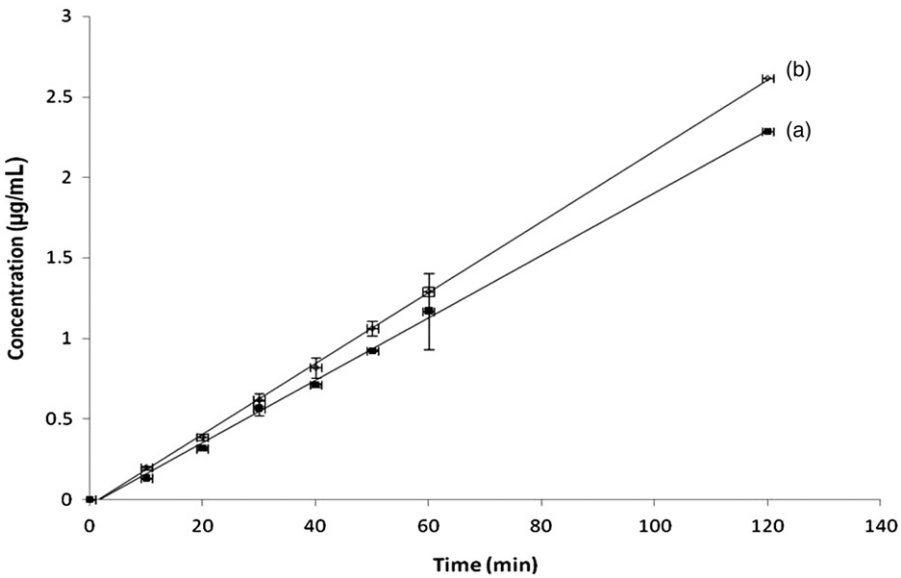

Figure 5. Average DIDR profiles ($n = 3$) of (a) DEF-ea (Method A) and (b) DEF-t-bw in deaerated MilliQ water, 37 ± 0.5 °C and 50 rpm.

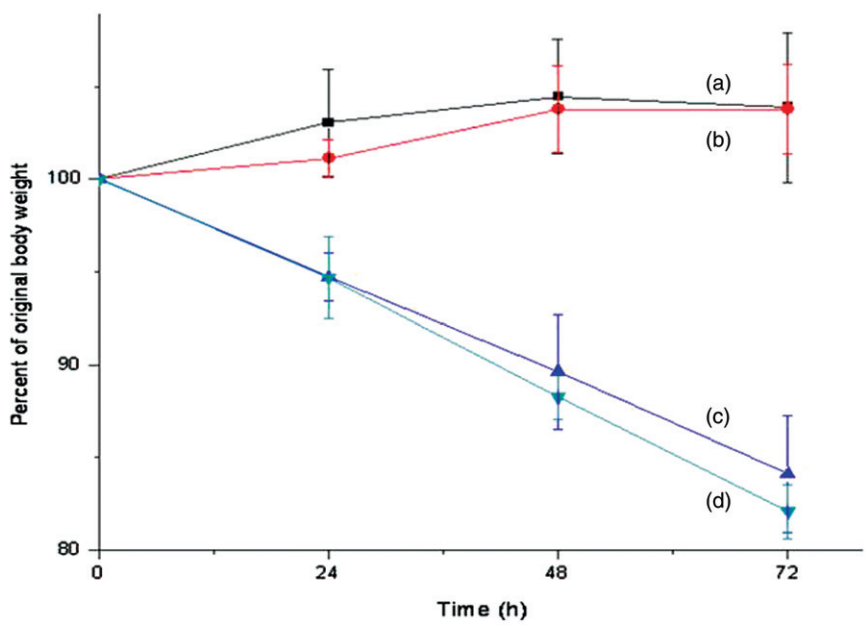

Figure 6. Animal weight variation for (a) control group ($n = 6$), (b) sham operated group ($n = 6$), (c) group ($n = 6$) treated with DEF-ea (Method A) implants and (d) group ($n = 6$) treated with DEF-t-bw implants.

three different solid-state forms: a crystalline one (DEF-1) that is obtained by crystallization from different solvents and conditions and being the raw material of choice for the preparation of commercial tablets; an X-ray amorphous hydrated form obtained by lyophilization from t-bu-water (DEF-t-bw), and an anhydrous amorphous/disordered form (DEF-g) obtained from manually grinding DEF-1. Although DEF-1 was the only form that could be isolated from the crystallization experiments performed, indicating a low likelihood for the existence of several anhydrous or solvated modifications of DEF, its conversion to the amorphous phases described in this work may be triggered during processing or formulation. However, the evaluation of the aqueous solubility, *in vitro* and *in vivo* DRs and solid-state stability of DEF-t-bw demonstrated that there is little solubility, DR or stability advantage associated with this amorphous form since it easily devitrified to DEF-1 by effect of mild compactation forces, temperature and humidity.

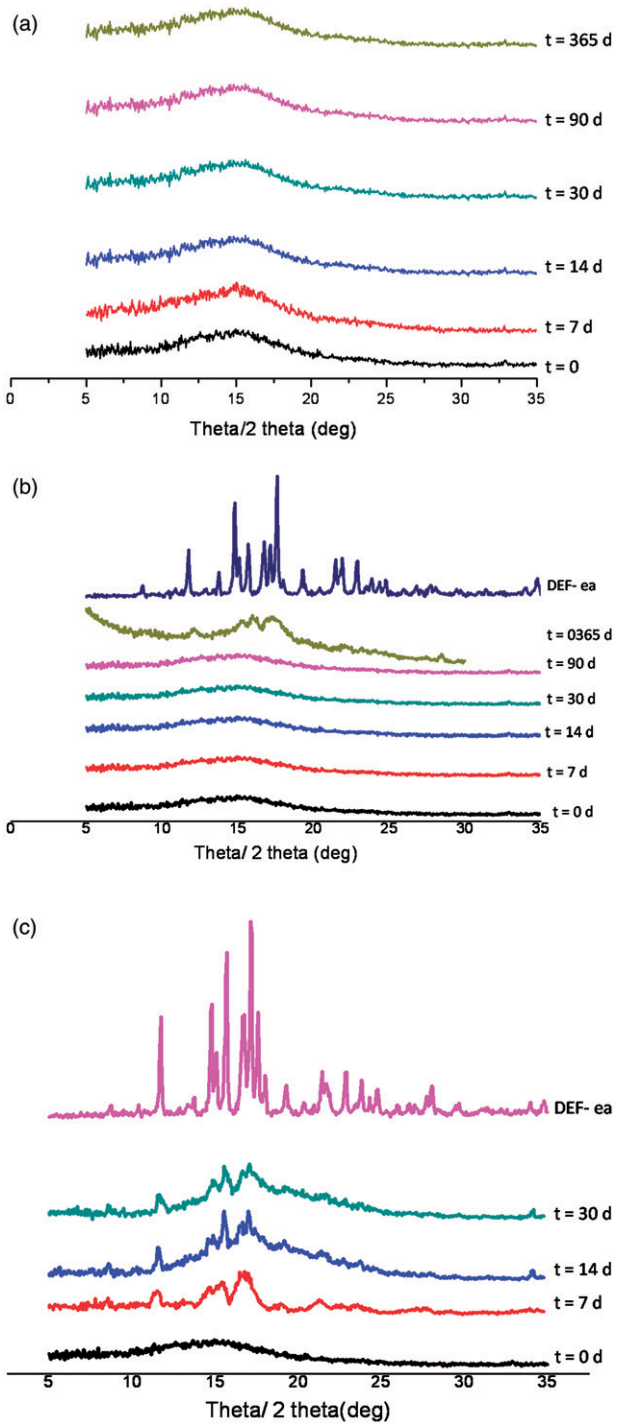

Figure 7. Powder X-ray patterns of DEF-t-bw after storage at different conditions. (a) At −20 °C/0% RH, (b) at 25 °C/0% RH and (c) at 40 °C/75% RH. The diffractogram of DEF-ea is given for comparison (top).

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Declaration of interest

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