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Isolation and characterization of a degradation product of deflazacort

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Deflazacort (DFZ) is an oxazoline derivative of prednisolone with anti-inflammatory and immunosuppressive activity. The aim of this study was to investigate and to identify the main degradation product of DFZ, and to evaluate the anti-inflammatory effect of both DFZ and its major degradation product (namely DDP1). DFZ was subjected to alkaline and acid degradation. In 0.1 N NaOH, DFZ was immediately degraded and 99.0% of product DDP1 was detected by high performance liquid chromatography (HPLC). The HPLC method was ideal to separate the primary and other minor degradation products and was carried out using C_{18} column, mobile phase consisting of water: acetonitrile: (60:40, v/v) with flow rate of 1.0 mL/min and detection at 244 nm. DDP1 was isolated and identified as 21-hydroxy deflazacort (21-OH-DFZ) by NMR, IR and LCMS. The *in vivo* pharmacological assays showed that both DFZ as 21-OH-DFZ are active in *in vivo* and *in vitro* inflammatory models, but 21-OH-DFZ is more potent than DFZ.

1. Introduction

Deflazacort (DFZ) (Scheme A) is an oxazoline (1-(1,16)-21-(acetyloxy)-11-hydroxy-2-methyl-5*H*-pregna-1,4-dieno [17, 16-*d*]oxazole-3,20-dione) derivative of prednisolone with anti-inflammatory and immunosuppressive activity (Joshi and Rajeshwari 2009). This glucocorticoid has been prescribed for the treatment of rheumatoid arthritis, asthma, and other applications such as myasthenia gravis, systemic lupus erythematosus, thrombocytopenic purpura, Duchenne muscular dystrophy, and kidney transplant patients (Angelini 2007; Biggar et al. 2001; Biggar 2006). It is a corticosteroid with a lower risk of side effects than other available steroids (Angelini 2007; Ferraris et al. 2007; Gonzalez-Castanheda et al. 2007; Joshi and Rajeshwari, 2009; Sousa et al. 2010). DFZ is currently available in tablets and oral suspension. An official method for determination of this drug in oral formulation has not been described yet and literature survey reveals few analytical methods reported for the quantitative estimation of DFZ (Gorog 2010). An HPLC method was developed for our group for the determination of DFZ in pharmaceutical dosage forms (Corrêa et al. 2007; Scremin et al. 2010). To verify the specificity of the method, forced degradation studies were performed (hydrolysis and oxidation). In these studies, we have observed the instability of DFZ under acidic and basic conditions. The stability of DFZ in solid state was also investigated by Cuffini et al. 2007 using differential scanning calorimetry (DSC), thermogravimetry (TG) and thin layer chromatography (TLC). However, in these studies, the degradations products were heights isolated has characterized. According to ICH guidelines, stress studies should be carried out on a drug substance to establish its inherent stability, leading to the identification of degradation products (ICH 2006). It is also important to evaluate the biological activity of an individual impurity or a given impurity profile, including degradation products. The hydrolysis in an aqueous environment of glucocorticoid that has a C-17 ester has been reported (Teng et al. 2003). Considering the few publications concerning stability studies of DFZ, the purpose of this work was to isolate and to identify the main degradation product of DFZ formed during hydrolysis conditions. In addition, anti-inflammatory effects of DFZ and major degradation product were evaluated by means of inflammatory models.

2. Investigations and results

In this study, hydrolytic stability of DFZ in aqueous solution was carried out under stress conditions. In preliminary forced stress testing, we have observed the instability of DFZ under acid and basic conditions, using a HPLC method developed for the assay of the DFZ in pharmaceutical dosage forms (Corrêa et al. 2007; Scremin et al. 2010). In this method, DFZ eluted at about 3.4 min and one detected unknown impurity eluted at about 2.8 min under acid and alkaline stress. To achieve better retention and resolution, the mobile phase composition of the our previously (80:20 (v/v), acetonitrile:water) method was modified to 60:40 (v/v) water:acetonitrile. In the optimized method the conditions were ideal to separate the primary and other minor degradation products and DFZ eluted at about 11.6 min. The method was validated according to ICH (2005) with a correlation coefficient >0.99 (in the range of 5.0–50.0 μg/mL), good specificity (resolution > 2), accuracy (mean recovery: 100,2%), and precision (RSD < 2%). In alkaline and acid conditions, the main peak was observed at a retention time of 4.7 min, and was namely as DDP1. Figure 1 represents the chromatograms showing the decomposition of DFZ in alkaline and acid conditions. In 0.1 N NaOH DFZ was immediately degraded and about 99% of product DDP1 was initially detected. DDP1 was degraded into other products after 24 h. In 0.1 N HCl relatively less degrada-

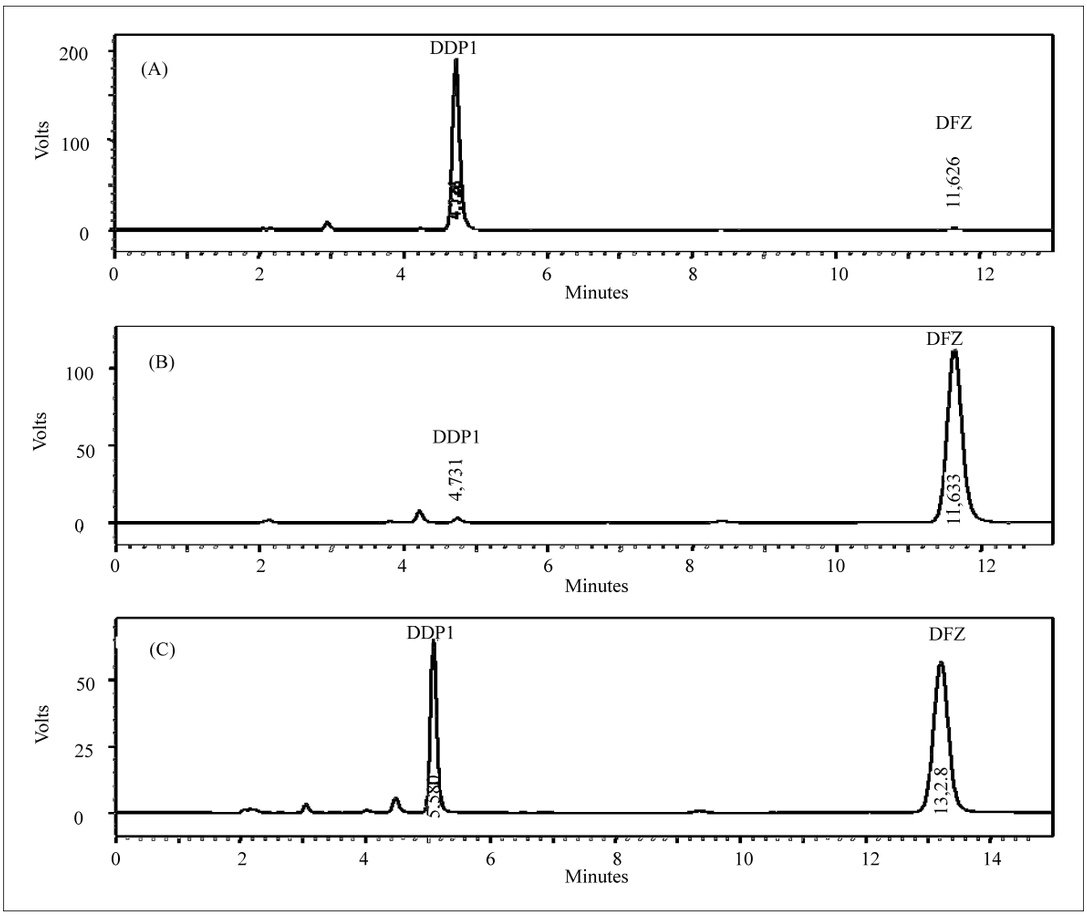


Fig. 1: Chromatograms showing decomposition of DFZ in alkali-induced degradation product (0.1 N NaOH), time zero (A); in acid-induced degradation product (0.1 N HCl) - time zero (B) and 24 hours (C), C18 (250 × 4.6 mm, i.d., 4 μm) column, mobile phase consisted of water:acetonitrile (60:40, v/v), applied at a flow rate of 1.0 mL/min, injection volume was 20 μL and elution of peaks was monitored at 244 nm

tion was observed, and about 39% of the major product DDP1 was detected after 24 h. Due to the extensive decomposition in alkaline solution, the DDP1 product was isolated from this condition by crystallization in methanol. So, the identification was possible. The structure of DDP1 was proposed according to the results obtained by NMR spectroscopy. The ^{1}H and ^{13}C NMR spectra were interpreted by comparing the chemical shifts of DFZ reference standard with those of degradation product, as shown in the Table. The NMR assignment of DFZ carbons is in accordance with data reported by Cuffini et al. (2007). The comparison of chemical shifts of DPP1 indicates that structure is similar, with a short modification. In the ^{13}C NMR spectrum of DFZ we observed the signal of a methyl (δ 20.3 ppm) and a carbonyl (δ 170 ppm). Differences in the relationship of the carbon spectrum between DFZ and DDP1 include the absence of signal of a methyl (δ 20.3 ppm) and of a carbonyl (δ170 ppm). The chemical shift of CH_2 at position 21 was less shielded on DFZ (δ 66.68 ppm) than on DDP1 (δ 65.67 ppm). In the proton NMR spectrum of DFZ, we observed the signals of four methyl (singlet) with displacement in 0.90, 1.38, 1.91, and 2.10 ppm. For DDP1 we observed a minor electronic effect of an OH group on C21, with doublets that are more shielded (δ 4.3 ppm). There are three methyl with displacement in 0.89, 1.37, and 1.86 ppm, and the singlet in 2.10 ppm disappears. The effect of an ester group on the DFZ molecule is higher than that of an alcohol group, and the ester is less shielded than the doublets (δ = 4.8 ppm). The IR spectrum of DFZ show main absorptions centered at 1749, 1730, and 1652 cm^{-1}, and were assigned to the carbonyl group in positions 20 and 22, and the cyclic α-β unsaturated 3-ketone (3-CO), respectively, as described by Cuffini et al. (2007). The spectrum of DDP1 revealed an absence of absorption at 1749 cm^{-1}, relating to carbonyl group in position 22.

In order to confirm the structure of DDP1, MS measurements were performed. The analysis was conducted using the direct insertion technique, without coupling with LC method. The mass spectrum of DFZ had an ion at m/z 442 (mass 441 + H), and m/z value for DDP1 was 399 (m/z 400). Results of LC-MS of DFZ (A) and DDP1 (B) are show in Fig. 2.

In order to evaluate the pharmacological effects of DFZ and DDP1, *in vivo* and *in vitro* studies were performed. In a paw edema model, 1 or 10 mg/kg of either DFZ or DDP1 (i.p.) significantly inhibited edema induced by carrageenan, with a maximal average inhibition, respectively, of 24 ± 2% and 29 ± 3%, or 39 ± 3% and 68 ± 4% after 360 min.

In a carrageenan-induced model of peritonitis, when the animals were treated with DFZ (1 mg/kg) or DDP1 (0.1, 1, or 10 mg/kg, i.p), a statistically significant decrease in the total number of cells in the peritoneal cavity was observed. DFZ (1 mg/kg) decreased the number of neutrophils to 46 ± 3%, while 1 or 10 mg/kg of DDP1 produced a mean inhibition of 65 ± 3% or 79 ± 4%, respectively, with an estimated IC50 value of 0.77 (0.70–0.89) mg/kg.

When assessed *in vitro*, treatment with 1, 10, or 100 μg/ml of either DFZ or DDP1 led to a decrease in nitrite concentration in the supernatant of RAW 264.7 macrophages stimulated with LPS with respective percentiles of 5 ± 1%, 21 ± 2%, and

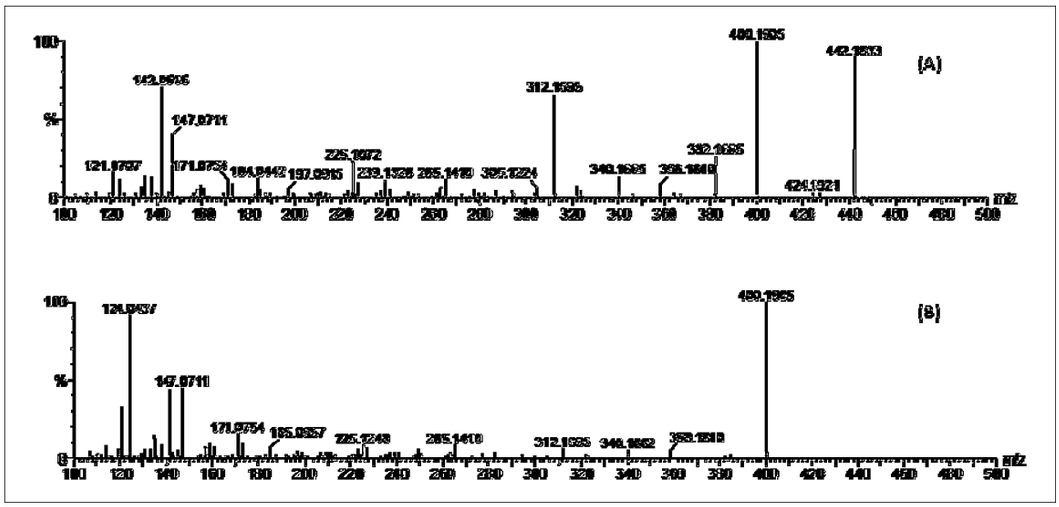


Fig. 2: LC-MS of DFZ (A) and DDP1 (B)

$24 \pm 2\%$, or $11 \pm 1\%$, $46 \pm 2\%$, and $61 \pm 3\%$, without interfere with the viability of cells, as shown by the MTT test.

3. Discussion

According to ICH (2006), pharmaceutical impurities are components found in a drug substance or drug product that are neither the drug substance nor excipients. Degradation products are a type of impurity (Li et al. 2008). The stress testing is the first part of the stability evaluation and can help to identify the likely degradation products, establish the degradation pathways and the intrinsic stability of the molecule. In general, these goals achieved in forced degradation studies should be conducted, in most cases, under conditions that induce thermal, alkaline, acid, oxidative and photolytic drug decomposition (Breier et al. 2008; Garcia et al. 2008; Mendez et al 2008). Teng et al. (2003) demonstrated that chemical stability of mometasone in aqueous systems was significantly dependent on pH, with maximum stability under weakly acidic conditions. Another study also demonstrated that halobetasol propionate is unstable in basic environment (Cravotto et al. 2007). DFZ, like these corticoids showed to be unstable under basic and acid conditions.

Table: Comparative ^{1}H and ^{13}C NMR assignments for deflazacort and DDP1

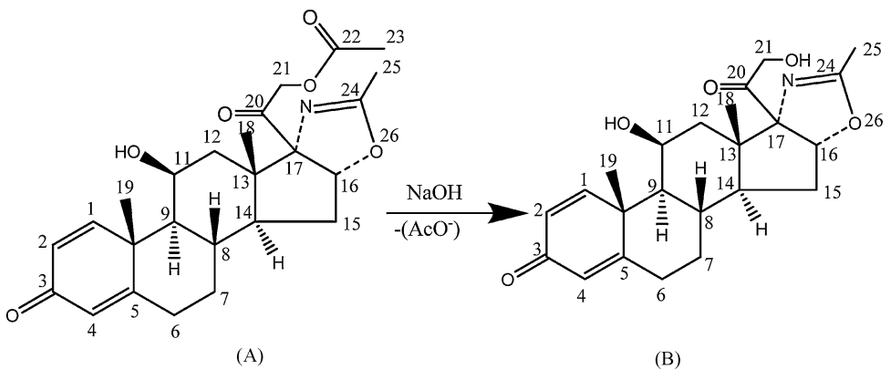
Position[a]	Deflazacort		DDP1	
	^{1}H (ppm), multiplicity	^{13}C(ppm)	^{1}H (ppm), multiplicity	^{13}C (ppm)
1	7.30 (d)	156.0	7.30 (d)	157.0
2	6.18 (d)	127.0	6.18 (d)	127.0
3	–	185.0	–	185.0
4	5.91 (s)	122.0	5.91 (s)	122.0
5	–	169.5	–	170.0
6	2.30 (m)	31.0	2.28 (m)	31.0
7	1.70 (m)	33.5	1.68 (m)	33.5
8	2.05 (m)	30.0	2.0 (m)	30.0
9	0.95 (m)	55.0	0.95 (m)	55.0
10	–	43.0	–	44.0
11	4.30 (s)	68.5	4.25 (m)	68.5
12	1.75 (m)	41.0	1.75 (m)	41.0
13	–	47.0	–	47.0
14	1,10 (m)	50.0	1.10 (m)	50.0
15	1.95 (m)	34.0	1.96 (m)	34.0
16	5.15 (m)	84.0	5.15 (m)	84.0
17	–	94.0	–	94.0
18	0.90 (m)	18.0	0.89 (m)	18.0
19	1.38 (s)	21.0	1.37 (s)	21.0
20	–	200.9	–	207.0
21	4.84 (dd)	66.6	4.30 (m)	65.7
22	–	170.0	–	–
23	2.10 (s)	20.3	–	–
24	–	165.0	–	165.0
25	1.91 (s)	14.0	1.86 (s)	14.0

s singlet; d. doublet; m. multiplet; dd. double doublet
[a] Refer structures (Fig. 1) for numbering

The degradation levels under hydrolysis conditions exceed the threshold indicated by ICH (2006) and the identification of DDP1 was necessary. The complete examination of the NMR, IR and MS spectra of the degradation product suggest deacetylation of DFZ. Degradation reactions of steroids primarily occur at the C-17 and C-21 side-chains, and this reaction is catalyzed by a proton, hydroxide, and trace metal ions (Amin et al. 1976). Studies have demonstrated that C-17 and/or C-21 esterified corticosteroids undergo hydrolysis in aqueous and biological media (Alman et al. 2004). DFZ is a C-21 esterified corticosteroid and the loss of acetyl group, resulted in 21-hydroxy-deflazacort (Scheme B). This compound is reported as an active metabolite of DFZ and its formation occurs when the DFZ is hydrolyzed by seric esterases in blood (Ifa et al. 2000; Santos-Montes et al. 1994). However, at this moment, the 21-hydroxy-deflazacort (21-OH-DFZ) had not been reported in the literature as a degradation product of DFZ from a stability study.

According to Ifa et al. (2000), DFZ is an inactive prodrug which is rapidly converted into the active metabolite 21-OH-DFZ after oral administration, and this metabolite has anti-inflammatory and immunosuppressive activities. To the best our knowledge, no comparative studies of anti-inflammatory activity of DFZ and 21-OH-DFZ by *in vivo* and *in vitro* anti-inflammatory models are reported in the literature.

From these results it is possible to conclude that DFZ is unstable under basic conditions, leading to the formation of 21-OH-DFZ. It is demonstrated in the literature that stability of mometasone furoate also is pH dependent (Sahasranaman et al, 2004; Teng et al. 2003). It was also shown, at least in part, that both DFZ and 21-OH-DFZ are active in inflammatory models, whereas 21-OH-DFZ is more potent. Our results agree with those described by Omote et al. (1994), indicating that while DFZ and 21-desacetyl-DFZ have stronger anti-allergic effects



Scheme: Chemical structure of DFZ (DFZ) (A) and its degradation product- DDP1: 21-hydroxy deflazacort (B)

than prednisolone, they seem to have little acute effect on mast cell degranulation or on chemical mediators at the receptor site.

4. Experimental

4.1. Materials and reagents

DFZ was obtained from Pharma Nostra (São Paulo, Brazil). Water was purified using a Millipore system Milli-Q Gradient. Sodium hydroxide, hydrochloric acid, and anhydrous sodium sulfate were analytical grade and were obtained from Vetec (São Paulo, Brazil). Dimethyl sulfoxide and tetramethylsilane deuterated were purchased from sigma. Chromatographic grade acetonitrile and methanol were purchased from Merck (São Paulo, Brazil). All chemicals used in *in vivo* and *in vitro* studies were acquired from Sigma Chemical Co. (St Louis, MO, USA).

4.2. Instrumentation

The HPLC system consisted of a Shimadzu LC-10 ADVP pump, an SPD-10AV VP UV-Vis Detector, an SCL-10 AVP system controller, and a degasser module; data were acquired and processed by Shimadzu CLASS-VP 5.032 software (Shimadzu, Kyoto, Japan). The column used was an RP18 (250 × 4.6 mm i.d., 4 μm). The mobile phase consisted of water:acetonitrile (60:40, v/v) at a flow rate of 1.0 ml/min. The injection volume was 20 μl, and the detection was set at a wavelength of 244 nm.

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker 500 MHz spectrometer using dimethyl sulfoxide (DMSO-d6) as solvent and tetramethylsilane (TMS) as internal standard. The infrared spectroscopy was recorded in Shimadzu FT-IR instrument using 1% (w/w) pressed discs with KBr.

A Waters HPLC system was used for the LC-MS analysis. The previously described chromatography conditions were employed, with minor changes; the mobile phase consisted of water with 0.1% formic acid and acetonitrile (60:40, v/v). An Analytical Biosystems, Q-TRAP was used for the mass spectrometric analysis. The ESI source was operated in positive ionization mode and its parameters were as follows: temperature, 300 °C; capillary voltage + 5500 V, DP + 50 V. Nitrogen was used as the nebulizer and collision gas. A Micromass Waters Q-TOF spectrometer was used for the ESI-MS/MS spectra under the following conditions: temperature 100 °C, 300 °C; capillary voltage + 3000 V, cone + 40 V. Nitrogen was used as the nebulizer gas and argon as the collision gas, collision 30 V.

4.3. Degradation procedure

For basic degradation, 12.5 mg of DFZ was transferred to a 50 mL volumetric flask and dissolved with 4.0 ml of acetonitrile. The volume was made up to volume with 0.1 N sodium hydroxide (NaOH). The flask was sealed and placed at 37 °C in bath, then cooled to room temperature at different times (0, 1, 2, 4, and 24 h). The pH of the solution was adjusted to neutrality by adding 0.1 N hydrochloric acid (HCl). The solution was filtered, diluted to 32 μg/mL and evaluated by HPLC. For acid degradation, the same basic degradation procedure was used, using 0.1 N HCl for degradation and 0.1 N NaOH for adjusted the pH to neutrality.

4.4. Isolation and characterization of degradation product

DFZ (100 mg) was dissolved in 4.0 mL acetonitrile followed by addition of 25 mL of 0.1 N NaOH. The pH was adjusted to 6.5–7.0 with 1 N HCl. The solvent was evaporated in a Savant SPD 1010 Speed Vac Concentrator, at ambient temperature. The residue was dissolved in methanol and filtered through quantitative paper. The methanol was evaporated in desiccators, and the identification of obtained crystal was carried out by NMR, IR and MS spectroscopy.

4.5. In vivo and in vitro assay

In vivo anti-inflammatory activity was evaluated by measurement of paw edema as described by Paulino et al. (2008), following Guidelines of Institutional Review Board of UNIBAN Ethical Committee 047/2009. *In vitro* assay was performed by nitric oxide determination and cell viability quantification as described by Paulino et al. (2006).

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