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Pharmaceutical preformulation studies and paediatric oral formulations of sodium dichloroacetate

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ABSTRACT

The purpose of this study was to develop liquid and solid paediatric formulations of sodium dichloroacetate (DCA) for the treatment of congenital lactic acidosis (CLA). In this work preformulation studies on the active molecule were performed to identify those physico-chemical properties of the drug relevant to the design of the dosage forms and their process of manufacture. TGA and DSC analysis suggested that sodium DCA was very hygroscopic. HPLC and NMR analysis showed that the compound was widely stable in aqueous solutions at 25 and 40 °C at all the pH values studied. Based on these results, sodium DCA was formulated as palatable solutions containing sweetener, viscosity enhancer and flavoring excipients tolerated by paediatric patients affected by CLA. The developed liquid formulations resulted chemically stable at 25 and 4 °C over three months. In use-stability tests showed no chemical degradation and microbiological contamination over one month. Oral tablets of sodium DCA were prepared by molding technique as an alternative and more practical formulation, easier to administer for caregivers than the liquid one. Technological assays (reported in the European Pharmacopeia) showed that oral tablets disaggregated quickly within 3 min at 25 °C in water, thus they were classified as orally disintegrating tablets. Preformulation studies provided a set of parameters against which detailed formulation design could be carried out. Formulation studies showed that the developed dosage forms achieved adequate stability, producibility and patient acceptability.

1. Introduction

Sodium dichloroacetate (DCA) has been administered for decades as an investigational drug for the treatment of several cardiovascular and metabolic disorders, such as ischemic heart disease, hyperlipidaemia, diabetes mellitus and lactic acidosis and more recently as a potential anticancer agent (Stacpoole, 1989). In particular, sodium DCA has been widely used for the acute and chronic treatment of acquired (Stacpoole et al., 1984, 1992) or congenital lactic acidosis (Barshop et al., 2004; Berendzen et al., 2006; Duncan et al., 2004; Stacpoole et al., 1997, 2006, 2008), for which it was designated as orphan drug by the Food and Drug Administration (FDA) ("FDA U.S. Food and Drug Administration"). The main reason behind its use is the ability to stimulate the activity of pyruvate dehydrogenase complex (PDC) by inhibiting the kinase involved in the phosphorylation of pyruvate dehydrogenase (PDH), thereby locking the enzyme in its non-phosphorylated active state (Stacpoole, 1989) (Fig. 1). Consequently, DCA accelerates the aerobic oxidation of glucose, pyruvate, and other molecules, such as lactate and alanine, that are in equilibrium with pyruvate, to acetyl coenzyme A (Acetyl-CoA) thus reducing tissue and circulating levels of lactate. Therefore, this compound might be particularly effective to treat patients affected by congenital lactic acidosis (CLA) due to a deficiency of PDC by stimulating residual enzyme activity and, as a result, cellular energy metabolism.

DCA is rapidly absorbed following oral administration, crosses the blood-brain barrier and activates PDC within few minutes (Stacpoole, 1989). Indeed, open-label studies have revealed its effectiveness in reducing blood or brain lactate concentrations so as to improve the morbidity in some patients with defects in the PDC or the respiratory chain and their neurological status (Stacpoole et al., 1997). However, it has also proved that chronic oral administration of DCA could cause reversible peripheral neuropathy and hepato-toxicity (Ammini and Stacpoole, 2003; Stacpoole et al., 1998). On the other hand, recent randomized controlled trials followed by other open-label studies have shown that oral DCA (at a dose of 12.5 mg/Kg twice daily) is generally well-tolerated by the majority of young children exposed to a

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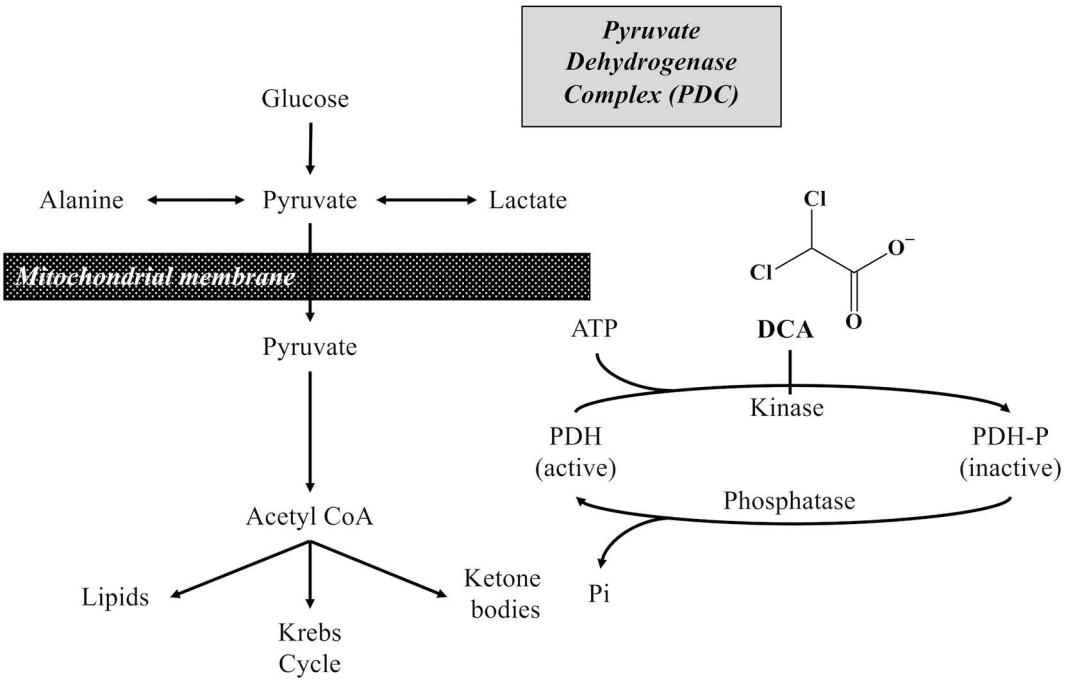


Fig. 1. Interaction of DCA with the subunit E1α of the PDH component of the multienzymatic complex (PDC). (ATP: adenosine triphosphate; Pi: inorganic phosphate).

continuous treatment for several months or years, without any significant side effects (Abdelmalak et al., 2013; Stacpoole et al., 2006, 2008).

To date, there is no univocal consensus on the therapeutic treatment of lactic acidosis because of the absence of a definite therapy supported by certain evidences. For instance, the integration with vitamins and co-factors such as thiamine is used in an attempt to stimulate the residual enzymatic activity (Kerr, 1995). A ketogenic diet based on high level of lipids would be an alternative source of Acetil-CoA and would seem to reduce the lactate levels although with limited benefits (Annese et al., 2013a; Wexler et al., 1997). Even though the therapy with sodium DCA is the only one that showed some significant clinical improvements (Stacpoole et al., 1997), today this active molecule is used exclusively as a lifesaver for the treatment of acidosis in which lactate concentrations reach critical values. Moreover, its unregulated use has spread after that some anti-cancer effects of DCA were demonstrated (Bonnet et al., 2007), even if direct preclinical evidences has been published only for non-small cell lung cancer, glioblastoma and breast cancers (Annese et al., 2013a; Dunbar et al., 2014; Garon et al., 2014; Michelakis et al., 2010).

In many countries of the European Union, including Italy, DCA-based extemporaneous formulations (especially liquid and capsules) are usually made by using non-pharmaceutical grade DCA. Indeed, lab grade DCA is sold legally online from various chemical manufacturers and is not suitable or approved for human use. DCA is a prescription drug in Canada, USA and through most of Europe. Thus, it cannot legally be sold as a medication unless it is under a doctor's prescription ("Medicor Cancer Centres, DCA (dichloroacetate) Frequently Asked Questions"). Moreover, in the literature, there is a lack of information regarding the physicochemical properties of sodium DCA.

In this study we will develop liquid and solid paediatric formulations of sodium DCA for the treatment of CLA, reserved for laboratory, such as a hospital pharmacy, and small-scale production, that could provide benefits over traditional dosage form. Preformulation studies on the active molecule will be perform to identify those physicochemical properties of the drug relevant to the design of the dosage forms and their process of manufacture. Based on these results, sodium DCA will be formulated as stable palatable solutions containing excipients tolerated by paediatric patients and orally disintegrating tablets (ODTs) of sodium DCA as an alternative and more practical formulation, easier to administer for caregivers than the liquid one. All the formulations will be manufactured with specific characteristics (i.e. taste, volume/size, shape, disintegration time) that could be more practical and enhance acceptability and adherence of medication in these paediatric patients. Moreover, the screening and careful selection of excipients will be a critical step in paediatric formulation development as certain excipients generally acceptable in children formulations, are not appropriate for children affected by CLA, e.g. saccharose in oral liquids formulations.

2. Materials and methods

2.1. Materials

Sodium DCA (98%) and (≥99%) were purchased from Sigma-Aldrich (Milan, Italy) and Curaltus UAB (Vilnius, Lithuania), respectively. Hydrochloric acid (HCl) 37% (ACS reagent), acetic acid ACS reagent (≥99.7%) sodium acetate (99%), NaH_2PO_4 anhydrous, Na_2HPO_4 anhydrous, Tris-HCl (> 99%), sodium chloride (NaCl), deuterium oxide (D_2O), were purchased from Sigma-Aldrich (Milan, Italy) and used without purification. Sucralose, polyvinylpyrrolidone ((PVP) Ph.Eur.-USP), "Mascagni" vehicle for oral solution and orange, vanilla, tropical and berries fruits flavors were obtained from Farmalabor SRL (Canosa di Puglia, Italy). Deionized water was used to prepare all samples for the high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) analysis. Highly purified water was used to prepare liquid formulations. Methanol HPLC grade was purchased from Sigma-Aldrich and used as external reference for NMR analysis.

2.2. Preformulation studies

2.2.1. Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA)

DSC measurements were performed on a Mettler Toledo DSC 822e Stare 202 system (Mettler Toledo, Greifensee, Switzerland) equipped with a thermal analysis automatic program. About 8 mg of each sample were placed in an aluminum pan (of 40 mL capacity and 0.1 mm thickness), press-sealed with a perforated aluminum cover and the temperature ramped at a rate of 5 °C/minutes (min) from ambient temperature to 220 °C (Cutrignelli et al., 2008). TGA experiments were performed on a TGA (Pyris 1 Perkin Elmer, Milan, Italy) instrument. The samples (~10 mg) were analyzed under dry nitrogen atmosphere in platinum crucibles at a heating rate of 10 °C/min from 30 °C to a final temperature of 350 °C.

2.2.2. Evaluation of hygroscopicity

The hygroscopicity of sodium DCA was assessed using the method reported by Callahan and co-worker with slight modifications (Callahan et al., 1982). Equilibrium moisture content (EMC) was gravimetrically determined by placing weighted samples of sodium DCA (0.277 g) in two open and tarred petri-plates. Then, the samples were placed in the climatic chamber (Climacell, MMM Medcenter-Munico Germany) at 95% RH, and 25 °C rather than in a close desiccator containing a saturated salt solution as provided in the Callahan's test. After 7 days of storage at controlled temperature and humidity, the samples were removed from the climatic chamber and weighted by a calibrated analytical balance obtaining the final equilibrium weight.

The initial moisture content (A) of the samples was determined by TGA in the same conditions previously described. Initial moisture content of the samples was used to calculate P (% dry basis), and EMC values were calculated from P using Callahan's equations Eqs. (1) and (2) and represented as mean.

$$P = \frac{\left[\left(W \times \frac{A}{100}\right) + B\right] \times 100}{W - \left[W \times \frac{A}{100}\right]} \quad (1)$$

$$EMC = \frac{P}{P + 100} \times 100 \quad (2)$$

where, P is the % moisture dry basis, W the initial sample weight in grams, A the % moisture at start, B the weight change at equilibrium in grams and EMC the equilibrium moisture content.

2.2.3. Preparation of test solutions

Sodium DCA solutions (~1 mM) were prepared using buffers tris (pH 9), phosphate (pH 7.4), and acetate (pH 4.6) at three different concentrations (10 mM, 25 mM and 50 mM) and HCl 100 and 1 mM, with ionic strength adjusted to 0.15 with NaCl. The exact concentrations of the DCA solutions were confirmed at time zero by HPLC (through a calibration curve) for all studies. The pH of each sample was measured at ambient conditions by using a pH meter (SevenExcellence, Mettler Toledo) equipped with a pH combination polymer electrode (InLab Expert Pro, Mettler Toledo).

2.2.4. HPLC method and stability studies in solution

The stability of sodium DCA was investigated in buffered aqueous solutions at pH 1.3, 3, 4.6, 7.4, and 9.0 at constant ionic strength (I = 0.15), as previously described. The resulting solutions were stored in glass vials at 25, 40, 65 and 80 °C in a climatic chamber (Climacell, MMM Medcenter-Munico Germany) for various time (up to 6 months) and their stability was monitored by HPLC following a method already reported (Lopalco et al., 2016a; Lopalco and Stella, 2016). An Agilent 1260 Infinity quaternary LC VL system equipped with a variable wavelength detector and with openLab DCS software was used. A Phenomenex Synergi 4 mm Hydro-RP 80 Å 250 × 4.6 mm^2 column thermostated at 40 °C was used. An isocratic separation was performed using 25 mM NaH_2PO_4 (pH 3) as mobile phase at a flow rate of 0.7 mL/min. The UV detection was carried out at 220 nm. An injection volume of 20 µL was used in all experiments.

2.2.5. Determination of pK_a of DCA by ^1H NMR experiments

Acid dissociation constant (K_a) for DCA was determined from chemical shift/pH ^{1}H NMR titration by adapting an already reported procedure by us (Lopalco et al., 2016b). Sodium DCA was dissolved in a volume of 0.6 mL of solvent (9:1, v/v, H_2O/D_2O) obtaining an initial concentration of 0.15 M. The pH of the sodium DCA solution was adjusted to the desired value by addition of concentrated HCl such that the final ionic strength was 0.15 with NaCl. The pH of each sample was measured using a pH meter (SevenExcellence, Mettler Toledo) equipped with 3 mm electrode (InLab Micro, Mettler Toledo). All spectra were acquired using 5 mm NMR tubes. No correction was made for the deuterium isotope effect. The samples resulted stable during the analysis and no variation in spectra and pH values was observed when the runs were repeated. One dimensional ^{1}H NMR spectra were recorded on Varian Mercury 300 MHz instrument at 25 °C. Chemical shifts (δ) were referenced to the internal standard methanol fixed at 3.31 ppm. The pH-titration curve was fitted to the Eq. (3) using GraphPad/Prism 6, Version 6.01 (©1992–2012 GraphPad Software, Inc.):

$$\delta_{obs} = \frac{[H^+]\delta_{HA} + K_a\delta_{A^-}}{[H^+] + K_a} \quad (3)$$

where δ_{HA} and δ_{A^-} are the chemical shifts of protonated form (HA) and deprotonated form (A^-) of DCA, and K_a is the acid dissociation constant.

2.2.6. Characterization of the degradation products in H_2O solutions by ^1H-, ^{13}C-NMR and heteronuclear multiple bond correlation

Solutions of sodium DCA (150 mM) in H_2SO_4 were stored at 80 °C in the climatic chamber and their stability was monitored by ^{1}H and ^{13}C-NMR experiments in the presence of 10% of D_2O. One dimensional ^{1}H and ^{13}C NMR spectra were acquired on Varian Mercury 300 MHz spectrometer at 25 °C (Caruso et al., 2017; Lopalco et al., 2016b). Chemical shifts were expressed in parts per million (ppm) with respect to D_2O signal for proton and to methanol for carbon. Heteronuclear multiple bond correlation (HMBC) spectra were recorded on Varian Mercury 500 MHz spectrometer at 25 °C. The following parameters were used for 2D ^{1}H- ^{13}C-HMBC experiment: number of scans, 32, number of complex data points (experiments) in F1, 16,348; number of complex data points in F2, 1202; sweep width in F1 and F2, 240 and 16 ppm, respectively; spectrometer offset for ^{1}H and ^{13}C, 6 and 110 ppm, respectively; interscan delay, 1.0 s (Lopalco et al., 2016a). Data were processed with the software Agilent NMR software VnmJ Datastation 4.2. Methanol was employed as internal standard for 2D-NMR spectrum.

2.3. Formulation studies

2.3.1. Oral liquid formulations

2.3.1.1. Palatability. Liquid formulations of 9.5% sodium DCA were prepared using sucralose as sweetener and different flavors such as orange, vanilla, tropical and berries fruits. The palatability of the test formulations was assessed by four adults, experienced in taste evaluation. Smell, taste, after-taste and mouthfeel were independently evaluated by the taste panel. A taste scale from 0 (unpleasant taste) to 3 (pleasant taste) was used to assess the palatability of all DCA solutions.

2.3.1.2. Preparation procedure. After the palatability evaluation, only three liquid formulations of 9.5% sodium DCA were prepared. Sodium DCA (9.5 g) was solubilized in 100 ml of highly purified water and the resulting solution was divided into 3 parts to which it was added respectively: i) 0.04% sucralose; ii) 0.04% sucralose and 0.04% orange

flavoring; iii) 0.02% sucralose, 0.25% hydroxyethyl cellulose, 0.09% citric acid, 0.09% sodium citrate dihydrate and 0.18% potassium sorbate ("Mascagni" vehicle for oral formulation). Only the latter preparation was kept under magnetic stirring at room temperature (r.t.) overnight to obtain a clear solution.

2.3.1.3. Long-term stability. The stability studies of the liquid formulations were performed using 500 mL amber glass bottles, with a diameter bottle of 76.8 mm, diameter headspace of 28 mm (conformed to EP and USP) and sealed with a polypropylene black screw cap (Farmalabor, s.r.l.). The influence of temperature and of packaging material on long-term stability of DCA formulations was investigated. The three formulations were stored in the climatic chamber at 25 ± 2 °C under controlled relative humidity (60% ± 5% RH) and at 4 ± 2 °C in glass containers. The stability of DCA in each formulation was monitored by HPLC for three months following the same method used for the DCA stability studies in solution (see "*HPLC method and in solution stability studies*" section). The pH of all formulations was measured immediately afterwards their preparation and during the stability tests.

2.3.1.4. In-use stability. An in-use stability test was performed on the three liquid formulations based on two-times daily dosing schedules. The containers were stored at r.t. and 4 ± 2 °C and twice-daily removed from the counter/fridge to be exposed to air, light and ambient temperature for 5 min at every dosing simulation. Samples of 7 ml were withdrawn. After 30 days, aliquots of the remaining formulations were analyzed by HPLC to investigate the chemical and physical stability. Microbiological stability was tested in accordance with the plate-count methods reported in the section "Microbiological examination of non-sterile products" of the European Pharmacopeia (Eur.Ph.) 9.0. ("Microbiological examination of non-sterile products: total viable aerobic count").

2.3.2. Orally disintegrating tablets (ODTs)

2.3.2.1. Preparation of tablets. Tablets were prepared by molding technique mixing sodium DCA powder with 1% sucralose and moistening the resulting powders mixture with a hydroalcoholic solution. For the preparation of 30 tablets, 9.9 g of sodium DCA and 0.099 g of sucralose were put in a mixing tool in order to obtain a homogeneous powder. Considering the inevitable loss of material that may occur during the filling phase of the molds, slight excess (10%) of the total powder mixture has been weighted. The mixed powder was wetted with 1.3–1.5 mL of a 5% w/w PVP 90° hydroalcoholic solution to obtain a consistency in order to let the mass to stick to the spreader without crumble. At the end of wet massing, the damp powder was spread on the cavity plate using a stainless steel spatula with plastic handle. The excess product on the cavity plate was eliminated using a silkscreened spatula. The ODTs were dried at r.t. and stored in a sealed vial until use. Four batches of 30 tablets have been produced (Fig. 2). OPTIMA TABLET® 300 mg, stainless steel spatula with plastic handle and silkscreened Veralite® spatula were provided by Farmalabor, s.r.l.

2.3.2.2. Evaluation of tablets. The tablets from all the batches were evaluated for different parameters as reported in the Eur.Ph. 9.0 (Section 2.9).

2.3.2.3. Hardness. The crushing strength of the tablets was measured using Erweka type TBH 200 Tablet Hardness Tester. The test was carried out on 10 tablets selected at random from each formulation batch and the average reading was detected ("Resistance to crushing of tablets"). The hardness is measured in Newton (N).

2.3.2.4. Friability. Friability tests were performed by Erweka TA 100 friability tester. Twenty tablets were first dedusted, weighed, and placed in the friabilator drum and the equipment was rotated at 25 rpm for 4 min ("Friability of uncoated tablets"). Friability was calculated according to Eq. (4):

$$\%Friability = \frac{initial\ weight - final\ weight}{initial\ weight} \times 100 \quad (4)$$

2.3.2.5. In vitro disintegration test. The test was carried out on 6 tablets using Apparatus A (suitable for tablets that are not > 18 mm long) described in the Eur.Ph. (Erweka ZT3) ("Disintegration of tablets and capsules"). Distilled water at 37 ± 2 °C and 25 ± 2 °C was used as a disintegration media and the time was measured in seconds (s).

2.3.2.6. Weight variation. Twenty tablets taken at random from each batch, were weighed and the average weight was determined. Then, tablets were weighed individually, and the individual weight was compared with an average weight (average weight ± 10%) as reported in the section regarding good manufacturing practices of galenic preparations of the Italian Pharmacopeia (NBF, F.U. XII edition). According to this test, none of the 20 units must have a weight lower or greater than the two values identified.

2.3.3. Data fitting

Data fitting to determine k_{obs} values was performed using GraphPad/Prism 6, Version 6.01 (© 1992–2012 GraphPad Software, Inc.). Non-linear least-squares regression analysis with weighting was used to obtain best-fit values to the pH rate profiles of the k_{obs} values.

3. Results and discussion

3.1. Preformulation studies

Sodium DCA is commercially available as technical-grade powder. In the preformulation studies, batches of the compound provided by different suppliers were used and some critical issues were observed. In fact, in addition to some declared low percentages of impurities (such as monocloroacetate, chloroform, etc.), the pH of different aqueous solutions of sodium DCA batches at the same concentration (0.1 M) was different and below pH 7. Since sodium DCA is a salt consisting of one sodium cation and the conjugate base anion of the organic acid, the

Fig. 2. Molding technique: (A) filled molds with wet powder blend; (B) ejection of the tablets from the molds; (C) sodium DCA tablets.

dichloroacetate, the theoretical calculation of pH at that concentration should provide a value > 8, if one considers a K_a equal to 0.09. Several manufacturing processes of sodium DCA are reported in literature such as chlorination of monochloroacetic acid with chlorine ("Beilstein Online, 2002. Dialog Corporation, File 390. Available at: http://www.dialogweb.com/servlet/logon?Mode=1. Cary, NC, USA: Beilstein Chemidaten und Software GmbH.,") or catalytic dechlorination of trichloroacetic acid (Annese et al., 2013b; E.D. Morris, 1991; G. Koenig, E. Lohmar, 1986). Thus, it can be hypothesized that the powder is contaminated by HCl, not properly removed during the purification of DCA. This compound is sold legally online from various chemical manufacturers and is not suitable or approved for human use. Sodium DCA sourced for the treatment of lactic acidosis disease should be manufactured in a way that meets the requirements of the biopharmaceutical regulated manufacturing process.

In this study, we used the batch of sodium DCA that gave a pH value around 8, purchased from Sigma-Aldrich.

3.1.1. Solid state characterization of sodium DCA

Sodium DCA powder was characterized by DSC and TGA analyses. The thermogram of DCA shows a single exothermic peak approximately at 200 °C corresponding to its decomposition (Fig. 3A).

TGA curves of sodium DCA, analyzed by the first derivative, showed a decomposition pattern of two steps (Fig. 3B). At temperature lower than 100 °C untreated sodium DCA (the same sample analyzed by DSC) exhibits a weight loss of 9.52% probably due to a loss of water (black line, Fig. 3B). According to DSC thermogram (Fig. 3A), a second degradation process was observed at ~207 °C with a weight loss of 50.33%. In order to confirm the loss of water observed in the temperature range of 50–100 °C, sodium DCA was dried at 80 °C for 2 h. The thermogram of this sample (red line, Fig. 3B) doesn't show any weight loss until to 200 °C. Moreover, dried sodium DCA was maintained in a climatic chamber at r.t., under controlled relative humidity

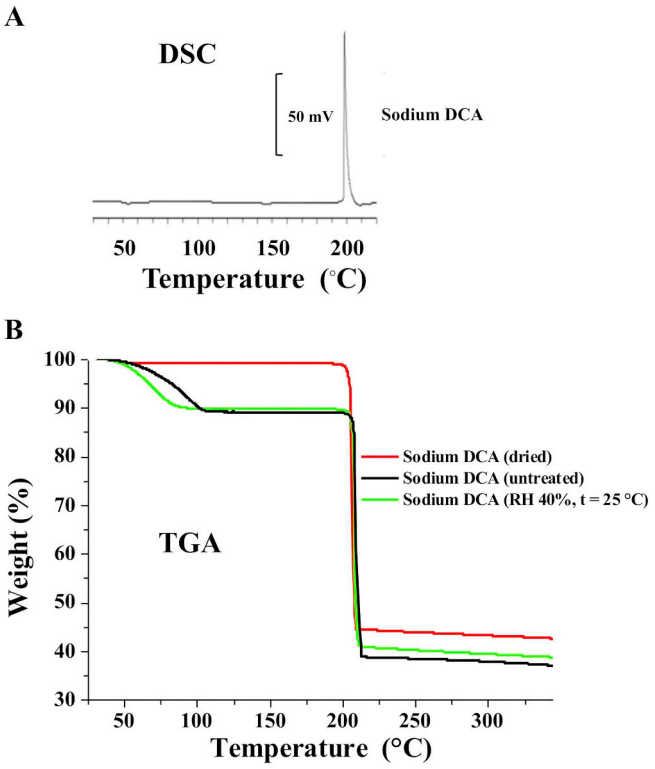


Fig. 3. DSC curve of sodium DCA (A) and TGA curves of untreated sodium DCA (black color), dried sodium DCA (red color) and treated sodium DCA (green color) (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(RH 40%) for two days. The TGA curve of this sample (green line, Fig. 3B) exhibits a faster weight loss (~10%) than the untreated sodium DCA at a temperature under 100 °C thus proving that the compound absorbs water also when the level of humidity is below that present in the atmosphere (Climatic zone II, subtropical and Mediterranean climates: 60% RH ± 5% RH, 25 °C ± 2 °C) (Futscher and Schumacher, 1972). The different velocity of this process (loss of water) can be ascribed to a different incorporation of water by the two samples suggesting that the compound should be kept in a tightly closed box and stored in a dry place.

Since a certain tendency of the powder to take up atmospheric water was highlighted using thermogravimetric analysis, we also evaluated the hygroscopicity by the conventional method (Callahan test) (Callahan et al., 1982). To estimate the water vapor content adsorbed by a solid, it's important to know the initial "dry" weight of the sample. Callahan et al. describe a method to calculate the initial percent moisture where the substances are dried for 2 h at 105 °C. As reported by Newman et al. (2008), the best option is to use the sample in its as received state, and independently determine initial water content by a method such as TGA or Karl Fischer titration. As previously reported in Fig. 3B (black color), the initial moisture content calculated by TGA is 9.52%. After Callahan's test, sodium DCA could be classified as a very hygroscopic compound because increase in moisture content is 18.62% at 40% RH and > 30% above 90% RH (EMC 53.55% at 94% RH).

3.1.2. Evaluation of stability in solution

The stability of DCA in aqueous solutions over a broad pH range between 1 and 9 at 25, 40, 65 and 80 °C was investigated by monitoring the disappearance of the parent peak by HPLC (Denora et al., 2007). All solutions of DCA stored at 25 and 40 °C resulted stable for at least 6 months. Thus, stress testing was also performed keeping samples at 65 °C as well as at 80 °C for 6 months. Fig. 4 shows the decline in DCA concentration (mM) plotted against time (days) of all solutions at different pH values heated at 65 and 80 °C. We assumed that the degradation of DCA followed a first-order kinetic which was described by a mono-exponential Eq. (5):

$$[DCA] = [DCA]_0 \times e^{-k_{obst}} \quad (5)$$

where $[DCA]_0$ is the initial concentration of the drug (1 mM), [DCA] is its concentration at time t, and k_{obs} is the observed first-order rate constant at each pH value, temperature and buffer concentration. By curve fitting the data to Eq. (5), it was possible to determine the values of k_{obs} under the experimental conditions. In all our experiments, we observed a faster degradation of DCA at 80 °C and at pH values higher than 3 (Fig. 4).

The impact of the buffer on the k_{obs} was also evaluated performing kinetic studies of sodium DCA at three different buffer concentrations, 10, 25 and 50 mM at pH 4.6, 7.4 and 9.0 at each temperature (Figs. 4 and 5). As it can be seen from the results in Fig. 4 no buffer catalysis was observed at pH values 4.6, 7.4 and 9, either at 80 °C (left) or at 65 °C (right).

The degradation of the drug was confirmed by HPLC technique. Fig. 6 shows that the hydrated form of glyoxylic acid, retention time (Rt) at 3.60 min, is the degradation product at pH 7.4 value.

The hydrated form of glyoxylic acid was the degradation product at all pH values studied.

The effect of chloride ions (Cl^-) on the degradation of DCA at 80 °C and pH ~1.3 was also investigated by monitoring the disappearance of the hydrogen signal of the drug by ^1H NMR (Fig. 7). The pH was adjusted to ~1.3 with H_2SO_4 rather than HCl. No NaCl needed to adjust the ionic strength of the solution since the concentration of sodium DCA used was 0.15 M. Under these experimental conditions, without Cl^-, the value of k_{obs} determined at pH ~1.3 was the same of that one observed in the presence of Cl^-. These findings confirmed that there was no effect of Cl^- on the rate constant of degradation of DCA under acidic conditions.

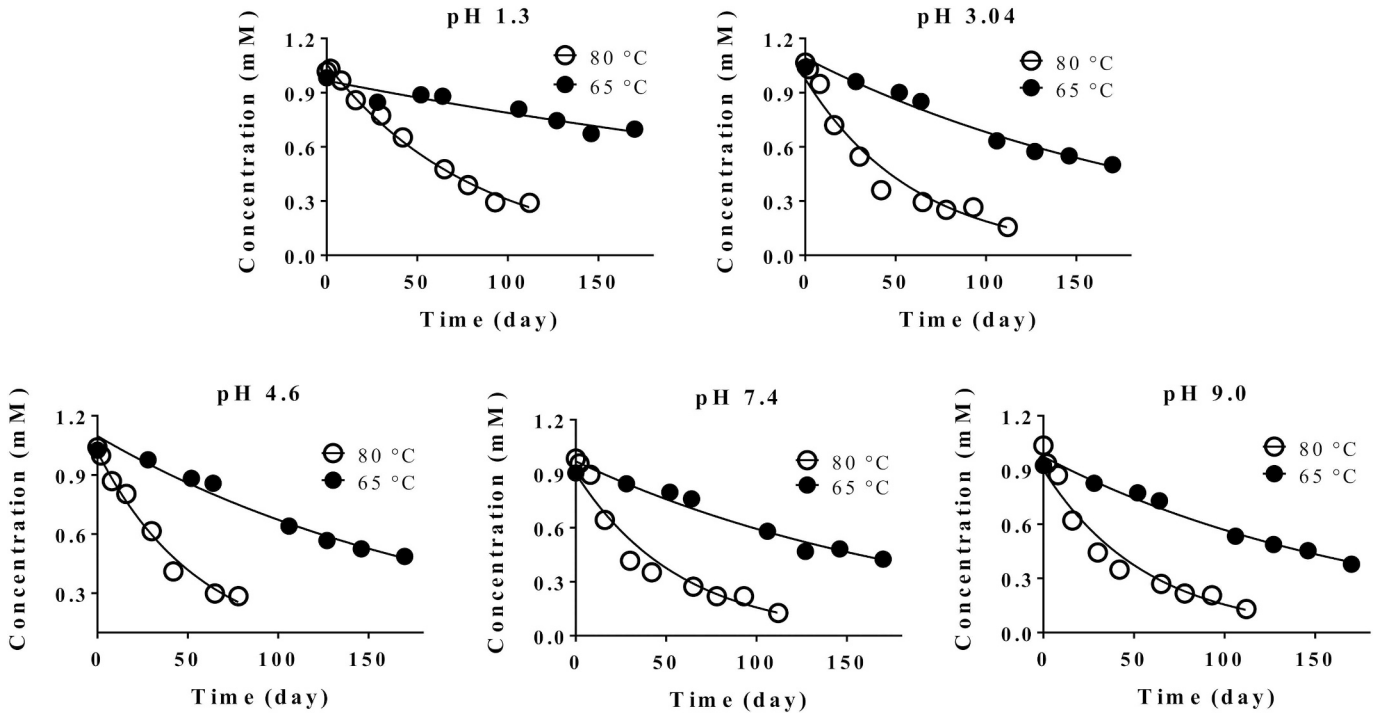


Fig. 4. Residual concentration of DCA in aqueous solutions at different pH values plotted as a function of time, assuming first-order loss. The reported plots were determined at 100 mM HCl (pH 1.3), 1 mM HCl (pH 3.04), 25 mM acetate buffer (pH 4.6), 25 mM phosphate buffer (pH 7.4) and 25 mM tris-buffer (pH 9.0), ionic strength 0.15 M NaCl at 80 and 65 °C. The results of each time point for each temperature were reported as mean of two experiments.

The degradation of the drug was also confirmed by ^{1}H-, ^{13}C- and 2D-NMR techniques (Fig. 7(I) and (II)). Fig. 7(I) shows that the ^{1}H NMR spectrum of DCA at time zero (t_0) displays a single peak at 6.16 ppm arising from methynic proton of DCA (top, on the left). After three months at 80 °C, a new peak at 5.30 ppm appeared (middle) and it corresponds to that of the methynic proton of the hydrated form of glyoxylic acid (bottom). The ^{13}C NMR spectrum of DCA at t_0 (top, on the right) shows two peaks at 169.84 ppm and 67.06 ppm, which are expected to be the signals for carboxylic carbon (B) and CH carbon (A) of DCA, respectively. The spectrum, recorded after 3 months at 80 °C (middle), exhibits other two more signals at 173.22 and 86.28 ppm corresponding to the carboxylic (D) and aliphatic carbon (C) of the degradation product, respectively. As it can be seen from Fig. 7(I), the hydrated form of glyoxylic acid shows the same two carbon peaks associated with the DCA degradation product thus confirming HPLC results.

2D-NMR investigation confirmed the presence of only one degradation product (Fig. 7(II)). Rt values and δ signals for the hydrated form of glyoxylic acid were confirmed by comparison a standard.

Two pH-rate profiles for k_{obs} were generated over a pH range of 1.3–9 at 65 and 80 °C (Fig. 8). As can be seen from this plot, k_{obs} results lower at pH values below 3. Above pH 3 the kinetic of degradation is faster than that one at pH 1.3 and pH-independent. This pattern could be ascribed simply to a different reactivity of the protonated and deprotonated forms of DCA which are in equilibrium in aqueous solutions as a function of pH (Scheme 1). The same trend is also observed at both temperatures with a faster degradation at 80 °C.

Thus, with the aim to prove our hypothesis, we determined the K_a of DCA at 25 °C by ^{1}H NMR technique. The δ of the signal arising from methynic proton (-CH) was followed by recording the spectra at different pH values (Fig. S1, left). The plot reported in Fig. S1 (right) displays the observed chemical shift (δ_{obs}) as a function of pH. The pK_a value of DCA, under our experimental conditions, calculated by fitting the data to Eq. (3) reported above, turned out to be 1.03 ± 0.06.

From the proposed mathematical model described in Scheme 1, an equation involving a simple ionization was derived to fit the pH-rate profiles of DCA at 65 and 80 °C.

The k_{obs} versus pH can be fitted using Eq. (6):

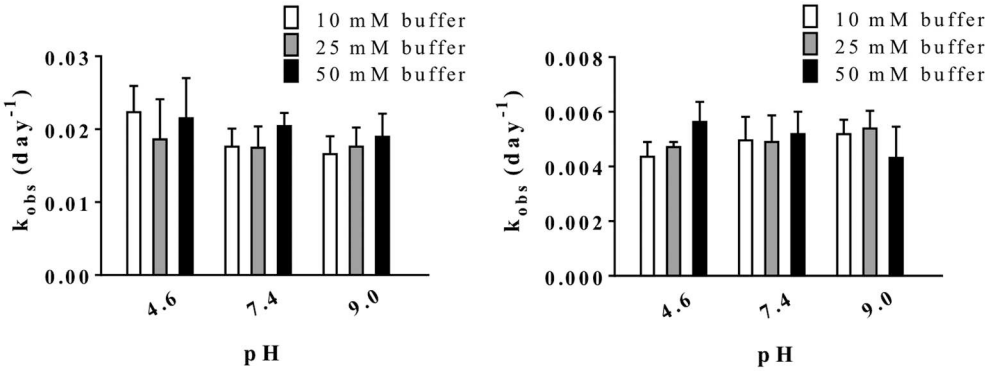


Fig. 5. Impact of buffer concentration on k_{obs} at 80 °C (left) and at 65 °C (right). The results at each buffer concentration were reported as mean of two experiments.

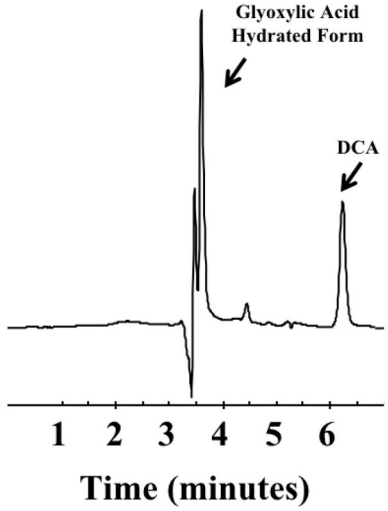


Fig. 6. A representative HPLC chromatogram of DCA and its degradation product (hydrated form of glyoxylic acid) in buffer phosphate 10 mM, pH 7.4, IS 0.15 M, after 2 months at 80 °C.

$$k_{obs} = k^A \times f_{HA} + k^B \times f_{A^-} \quad (6)$$

where k^A, k^B, f_{HA} and f_{A^-} are the kinetic rate constants and the mole fractions of the protonated (HA) and deprotonated form (A^-) of DCA, respectively. The mole fractions f_{HA} and f_{A^-} are described by Eqs. (7) and (8):

$$f_{HA} = \frac{[HA]}{[HA] + [A^-]} \quad (7)$$

$$f_{A^-} = \frac{[A^-]}{[HA] + [A^-]} \quad (8)$$

The mole fractions can then be expressed as a function of $[H^+]$ and the individual K_a, assuming that activity coefficients are unity:

$$f_{HA} = \frac{[H^+]}{[H^+] + K_a} \quad (9)$$

$$f_{A^-} = \frac{K_a}{[H^+] + K_a} \quad (10)$$

and, thus, Eq. (6) can be redefined by Eq. (11):

$$k_{obs} = k^A \times \frac{[H^+]}{[H^+] + K_a} + k^B \times \frac{K_a}{[H^+] + K_a} \quad (11)$$

At pH values above the pK_a of DCA the term $\left(k^A \times \frac{[H^+]}{[H^+] + k_a}\right)$ can be neglected, thus Eq. (11) can be simplified into Eq. (12):

$$k_{obs} = k^B \times \frac{K_a}{[H^+] + K_a} \quad (12)$$

Scheme 1 and the derived mathematical model can explain the degradation pathways of DCA in the pH range between 1.3 and 9 at 65 and 80 °C (Fig. 9). At pH values above 1 and below 3, k_{obs} was pH-dependent and Eq. (12) can describe the pathway of degradation of DCA. At pH above 3 (two units higher than the pK_a of DCA), the species presents in solution was only the deprotonated form A^- of the acid. From pH 3 to 9, when $[H^+] \ll K_a$, k_{obs} was constant and pH-independent. In this pH range, k_{obs} could be described by Eq. (13):

$$k_{obs} = k^B = \text{constant} \quad (13)$$

From the curve fitting two inflection points at 1.3 and 0.85 for the plot at 65 °C and 80 °C, respectively, could be observed. As one can see, these values were very close to the experimental pK_a determined by ^{1}H-NMR technique. The apparent pK_a value at 80 °C determined by curve-fitting was smaller than the experimental pK_a value determined by NMR technique at 25 °C (1.03). This could be attributed to a temperature-dependency of the equilibrium constant K_a, described by van't Hoff equation. Contrary, at 65 °C the pK_a value increased and was higher than the pK_a value determined at 25 °C. The last finding suggested that a more complex degradation pathway of DCA, than the proposed one showed in Scheme 1, probably involving intermediate species, could occur under those experimental conditions. In this work, it is not our object to study in deep the mechanism of degradation of DCA at higher temperatures.

In a study on the kinetics of degradation of chloramphenicol, Higuchi proved the hydrolysis of the drug at the amide linkage which yields an amine and a dichloroacetate ion (Higuchi and Bias, 1953). Subsequently, DCA ions undergo further hydrolysis giving off chloride ions in a pH-independent reaction. This type of hydrolysis has also been established by Kunze in an earlier study (Kunze, 1941). Here, we have demonstrated that, at elevated temperatures, DCA underwent the same hydrolysis reaction after a nucleophilic attack by water that leaded to the formation of the hydrated form of glyoxylic acid.

In an attempt to obtain specific information regarding the stability of DCA at 25 and 40 °C, the natural logarithm of k_{obs} values (lnk_{obs}) in the pH independent region were plotted versus the reciprocal of temperature expressed in Kelvin degrees (1/T) (Fig. 10). It was possible to obtain an Arrhenius plot (Fig. 10) using the k_{obs} values determined at 80 and 65 °C, and the data already reported in the literature by Kunze (Table 1) (Kunze, 1941). From the Arrhenius plot, we were able to extrapolate the k_{obs} and calculate the half-life ($t_{1/2}$) of DCA in aqueous solution also at 25 and 40 °C. The calculated values confirmed that DCA should be widely stable (Table 1). Energy of activation (E_a) values was 33 kcal and agreed with the values reported in the literature by Higuchi and Kunze.

3.2. Formulation studies

3.2.1. Oral liquid formulations

Preformulation studies have been very useful for the identification and development of new DCA oral formulations suitable for paediatric patients. The high stability of the drug in solution at 25 °C over a wide pH range allowed us to realize various liquid formulations. One of the most important issues in the development of medicinal products for paediatric patients is the most appropriate dosage form in relation to age. Indeed, different aspects related to the type of patient, such as age, pathology and compliance, have been considered in the choice of formulation. According to the European Medicines Agency (EMA) reflection paper on paediatric formulations, an oral liquid dosage form is generally the best applicable to administer systemic medication to infants and toddlers (1 month–2 years) and young children (2–5 years) ("Committee for Human Medicinal Products (CHMP) Reflection paper, 2006: Formulations of choice for the paediatric,"). Lactic acidosis can occur from birth so that a liquid is preferable to a solid formulation for patients up to 6 years of age. Further properties that need to be considered when designing a paediatric liquid preparation are dose volume (preferably ≤5 mL for children under 5 years) and use of child-friendly excipients ("Committee for Human Medicinal Products (CHMP) Reflection paper, 2006: Formulations of choice for the paediatric,"). Moreover, liquid formulations offer the advantage of dosing flexibility.

Based on a therapeutic plan adopted by a local paediatric hospital (Unità Ospedaliera Giovanni XXIII, Bari, Italy), we prepared different liquid formulations containing 9.5% DCA. In this way, a low dose-volumes (5–15 mL) of the preparation would be more appropriate for oral use in younger age patients affected by congenital lactic acidosis. A possible disadvantage of these formulations is the palatability, which represents one of the main elements of the patient's acceptance of a medicinal product. It is determined by the characteristics of the components (API and excipients) of a formulation. Different approaches might be utilized to obscure the unpleasant taste of APIs in paediatric oral dosage forms such as the use of masking agents. In our case,

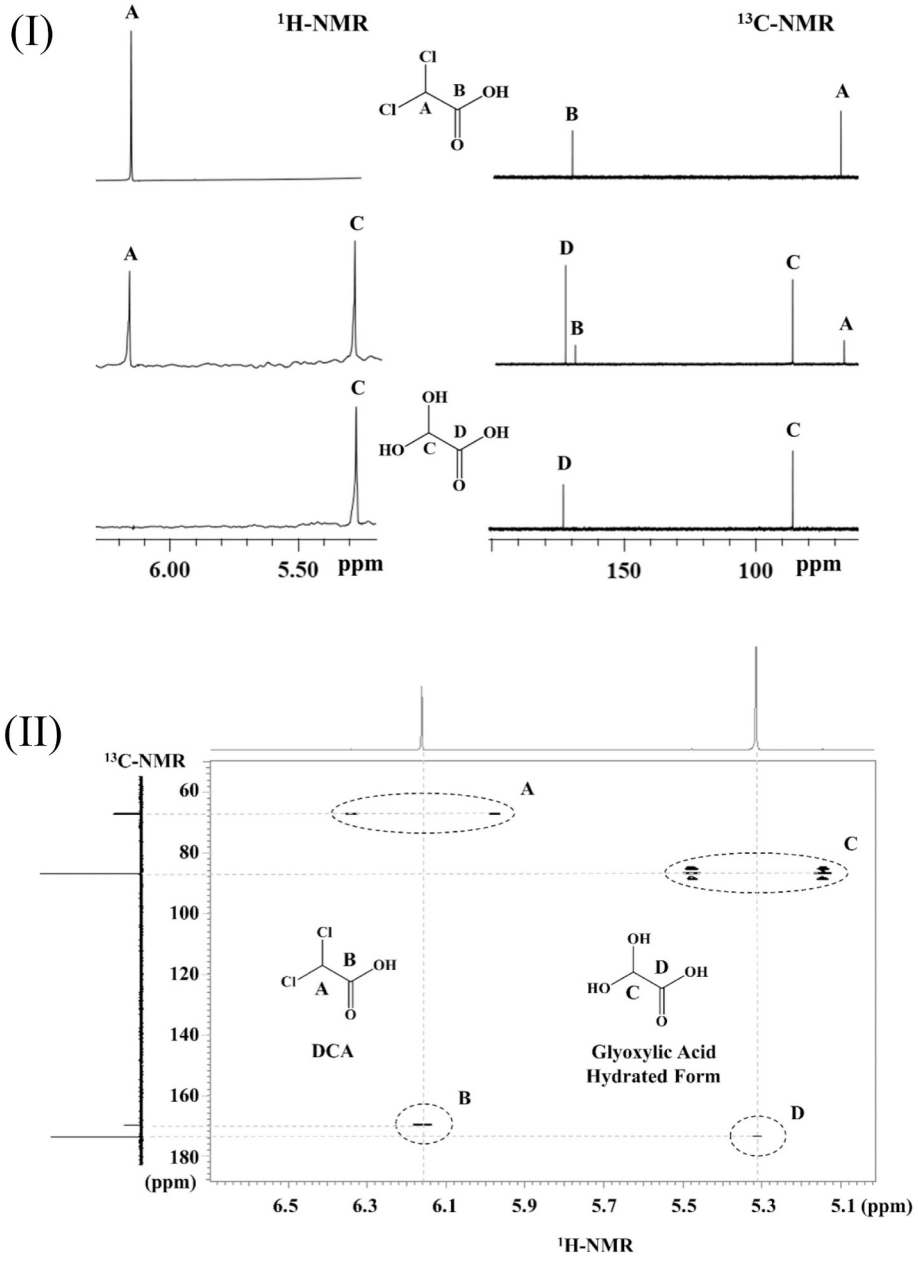


Fig. 7. (I) ^{1}H NMR (left) and ^{13}C NMR (right) spectra of DCA at pH ~ 1.3 (0.1 M H_2SO_4) at time 0 (top) and after 3 months at 80 °C (middle), and of glyoxylic acid at pH ~ 1.3 (bottom). (II) 2D ^{1}H- ^{13}C-NMR spectrum of DCA at pH ~ 1.3 (0.1 M H_2SO_4) after 3 months at 80 °C in H_2O:D_2O (9:1, v/v). The cross peaks displayed by HMBC were used to identify the structure of DCA and its degradation product (glyoxylic acid hydrated form), including the correlation of the δ of hydrogens and carbons separated of each other with two chemical bonds.

sweeteners and flavors were added to liquid formulations to mask the bitter-salty taste of the DCA. Among them, the most common excipients used in paediatric formulations (i.e. sucrose, fructose, lactose, aspartame, natural and synthetic polysaccharides) have been excluded because such substances, interfering directly or indirectly with the Krebs cycle, might create additional imbalances in patients already suffering from this illness. At this point it was decided to use sucralose because of its safety and sweetness (600-fold higher than sucrose) ("Commission European Parliament and Council Directive 94/36/EC of 30 June 1994 on colours for use in foodstuffs, 1994"). Sucralose is an artificial sweetener and sugar substitute, slightly absorbed, not metabolized in humans and excreted without transformations. Sucralose has no effect on carbohydrates metabolism, on blood sugar and insulin levels so that it is suitable for people with diabetes. The Acceptable Daily Intake (ADI) level for sucralose was set at 5 mg/kg body weight per day in the USA (U.S. FDA, 1998) and 15 mg/kg/day in the EU as recommended by Scientific Committee on Food of the European Commission (SCF, 2000) (WHO, World Health Organization, 1991). In our case, only 0.04% of sucralose, corresponding to a daily dose of 5.6 mg, was used in liquid preparations (F1 and F2, Table 2). With the aim of further masking the taste of the liquid formulations, some flavorings (orange, vanilla, tropical and berries fruits) were added to the formulations already containing sucralose and then, their palatability was assessed by a group of four adults. The taste evaluation results within the panel were consistent. Orange flavor was the preferred taste corrector for the bitter-salty taste of the DCA formulation followed by tropical fruits, berries fruits and vanilla. Thus, orange flavor was chosen and used in formulation 2. Moreover, a third liquid preparation was prepared using other excipients ("Mascagni" vehicle) to enhance its palatability and stability (F3, Table 2). The amount of sucralose was reduced to 0.02%

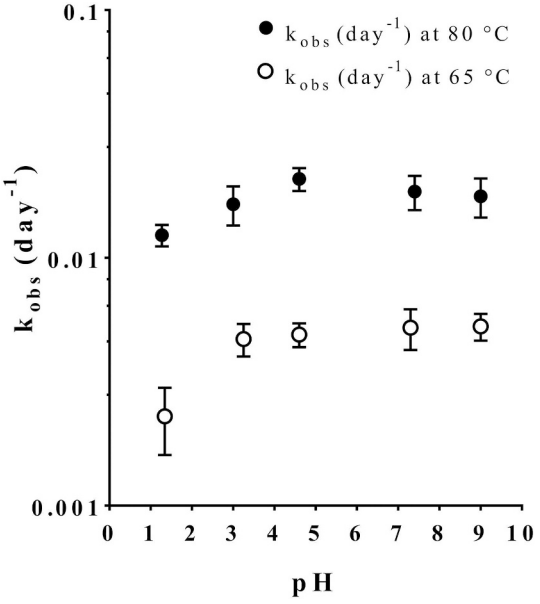


Fig. 8. pH-rate profile for the degradation of DCA at 80 and 65 °C in aqueous buffers between pH 1.3 and 9.0. Experimental values were determined at 10, 25 and 50 mM buffer concentration and 1 and 100 mM HCl and reported as mean value at each pH value.

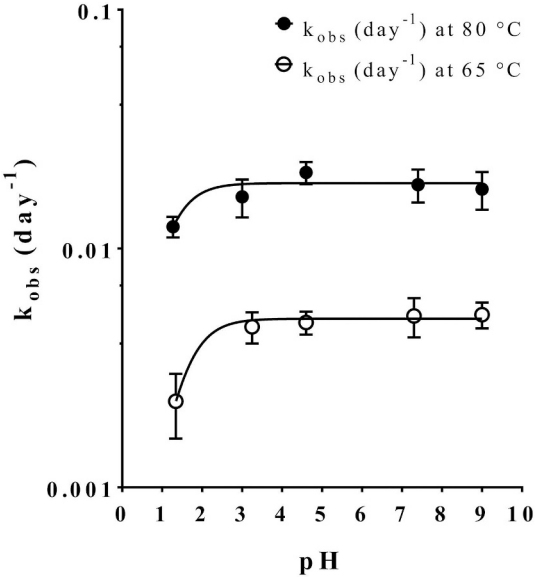
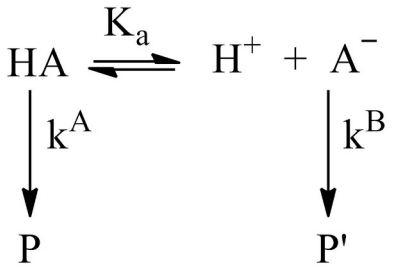


Fig. 9. The pH dependence of k_{obs} for the degradation of DCA at 80 °C and 65 °C in aqueous solutions between pH 1.3 and 9. Experimental values were determined at 10, 25 and 50 mM buffer concentration and 1 and 100 mM HCl and reported as mean value at each pH value. The solid lines were obtained by fitting the results to Eq. (12).



Scheme 1. Proposed degradation and ionization scheme involving both the protonated (HA) and deprotonated (A^-) forms of DCA in equilibrium in aqueous solution as function of pH. k^A and k^B are the kinetic rate constants of HA and A^-, respectively, P and P′ their degradation products (the protonated and deprotonated species of the hydrated form of glyoxylic acid, respectively) and K_a the acid dissociation constant of DCA.

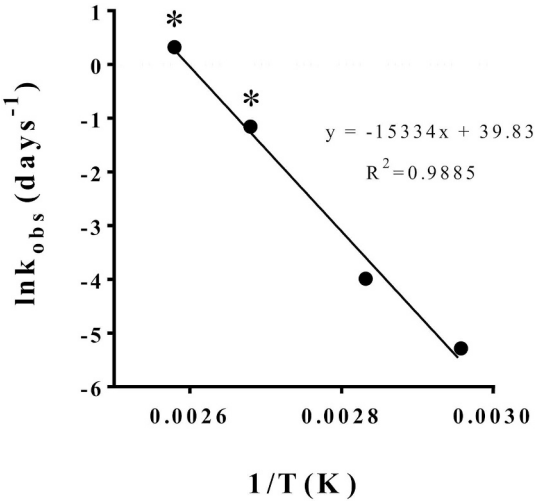


Fig. 10. Arrhenius plot for the degradation of DCA at pH 7.4. $\ln k_{obs}$ calculated and reported in the literature (*).

because of the addition of 0.25% hydroxyethyl cellulose chosen to give sufficient viscosity to the formulation such as to disadvantage the contact with taste buds. In this way, the bitter-salty taste of DCA is completely covered. Furthermore, 0.09% of citric acid/sodium citrate and 0.18% potassium sorbate were added to buffer and preserve the oral solution, respectively.

The unpleasant taste of sodium DCA was completely masked in all three formulations. Regarding the palatability evaluation by healthy volunteers, it is known that children experience different taste sensations than adults (Mennella and Beauchamp, 2008). In this stage of development, we considered a first screening by an adult tasting panel acceptable. A palatability assessment will be included in the clinical trial that will be performed with our formulations in congenital lactic acidosis paediatric patients.

To establish the best storage conditions, the stability of DCA formulations at 25 and 4 °C was evaluated by HPLC for three months. All the three formulations resulted chemically stable at both temperatures with a very high content of DCA (≥99.2%) (Table 3). In addition, a change in pH values from 6.8 to ~5 was found for F1 and F2 preparations. Instead, no pH variation was observed for the liquid formulation prepared with Mascagni vehicle probably due to the presence of citrate buffer (pH 4.8) in the vehicle. No changes in color and clarity were observed in any of the formulations. The packaging material did not influence the chemical degradation of sodium DCA.

Table 1
Rate constants (k_{obs}) and half-lifes ($t_{1/2}$) calculated and extrapolated (#) based on Arrhenius plot and reported in the literature (*).

Temp (°C)	k($days^{-1}$)	$t_{1/2}$ (days)
114.5	1.38*	0.50*
100	0.31*	2.20*
80	0.0185	37.5
65	0.0051	137
40	0.000105#	6600#
25	0.0000089#	77,882#

The samples of the three studied formulations remained stable during the in-use study and no visual changes were observed. The content of DCA did not decrease during the in-use study (data not

Table 2
Composition of the studied formulations, which were produced in batches of 100 mL and put into glass containers.

Composition of aqueous liquid formulations	% (w/v)
F1	
Sodium DCA	9.5
Sucralose	0.04
F2	
Sodium DCA	9.5
Sucralose	0.04
Orange flavor	0.04
F3	
Sodium DCA	9.5
Mascagni vehicle	
Sucralose	0.02
Hydroxyethyl cellulose	0.2
Citric acid	0.09
Sodium citrate	0.09
Potassium sorbate	0.18

shown). The total aerobic microbial count (TAMC) and total yields and molds counts (TYMC) were < 100 colony forming unit (CFU)/ml and < 10 CFU/ml, respectively, at 30 days of the in-use study in all samples. Moreover, no *Escherichia coli* were found in the samples. All the three formulations stored at 25 and 4 °C satisfied the acceptance criteria for aqueous preparations for oral use reported in the Eur.Ph. (Section 5.1.4.) ("Microbiological quality of pharmaceutical preparations"). Therefore, also the formulations without preservatives (F1 and F2) can be used over a period of one month even if the formulation F3 is the only that showed a stable pH over time.

3.2.2. Orally disintegrating tablets (ODTs)

Solid oral dosage forms such as tablets and capsules can offer advantages of greater stability, accuracy of dosing and improved portability over liquid formulations. The main limitation of solid oral formulations for paediatric use is the difficulty in swallowing of paediatric patients (dysphagia). Children aged lower than 6 years can learn to take solid oral formulations, particularly for chronic therapy thus, size of tablets or capsules should be as small as possible. In such scenario, orally disintegrating tablets (ODTs) can help in improving patients' compliance.

Therefore, we also developed ODTs of sodium DCA as an alternative and more practical oral formulation. We realized orodispersible, medium-sized tablets (< 10 mm) by molding technique using OPTIMA TABLET® 300 mg technology (Fig. 2). Molding technique is an old preparation method of tablets which represents today a valid alternative to the conventional tablet compression specially for pharmacists. Indeed, this technique allows producing small and medium batches of tablets for human use with personalized doses. This manufacturing process involves moistening the powder blend with a hydroalcoholic solution followed by pressing into mold plates (compression molding) and air-drying.

Four batches of thirty sodium DCA tablets each were produced and evaluated for different properties: hardness, friability, disintegration time and weight variation (Table 4).

Table 4
Properties of sodium DCA tablets. Hardness and disintegration time (at 37 °C) were reported as mean values ± standard deviation (SD).

Batch	Hardness ± SD (N)	Friability (%)	Disintegration time ± SD (s)	Weight variation (Italian Ph.)
1	60.0 ± 0.50	3.0	58 ± 2.5	Passed
2	79.2 ± 0.29	2.5	56 ± 3.4	Passed
3	60.0 ± 0.6	2.0	59 ± 2.0	Passed
4	78.0 ± 0.29	3.0	58 ± 4.0	Passed

The tablets must be hard enough to withstand mechanical stress during shipment and handling by the consumer. In particular, oral uncoated tablets normally have a hardness of 40–80 N. In our case, we obtained tablets with a suitable hardness (60–80 N). Also friability is a property that is related to the resistance to crushing of tablets. According to European Pharmacopeia, a maximum loss of weight not > 1% is considered acceptable for most products. Even though, sodium DCA tablets friability ranged between 2 and 3%, we considered these values acceptable since our goal was to develop tablets that disintegrate rapidly. Moreover, the high friability could be ascribed to the molding compression which produces less compact tablets than compressed tablets.

The in vitro disintegration time was determined by disintegration test apparatus. European Pharmacopeia had used the term of orodispersible tablets to define "uncoated tablets intended to be placed in the mouth where they disperse readily within 3 min before swallowing". Sodium DCA tablets disintegrated in < 1 min at 37 °C. Since ODTs can be administered with water, we repeated the disintegration test at 25 °C in accordance with that required for "soluble tablets" reported in the Eur. Ph. Also in this case the test was satisfied because tablets disaggregated within 3 min.

Furthermore, all batches of tablets passed weight variation test for solid galenic preparations reported in the Italian Pharmacopeia. Tablets with an average weight of 302 mg were obtained and none of the 20 tablets of each batch that underwent the test were above or below the calculated limits (± 10% of the average weight). The uniformity of dosage units test has not been performed because our tablets are mainly made of active principle (sodium DCA).

The proposed solid oral dosage form ODT can represent an alternative formulation to the liquid one. The tablets prepared using molding technique is reserved for laboratory, such as a hospital pharmacy, and small-scale production. ODTs can provide many benefits over traditional dosage forms for paediatric patients, such as rapid disintegration within the oral cavity upon contact with saliva and reduction in the risk of choking. ODTs can be taken without the need to swallow the tablet whole and does not require water. Regarding dosing error, liquid formulations require calculation and measurement of the dose volume whereas ODTs are available in the appropriate dose and do not need further manipulation. Moreover, according to healthcare professionals opinions, acceptability and adherence of medication in

Table 3
Results from the chemical stability studies at 3 months and from microbiological stability test after 1 month of all three formulations at 25 °C. The same results were found at 4 °C (data not shown). (CFU: colony forming unit; TAMC: total aerobic microbial count; TYMC: total yields and molds counts).

	F1		F2		F3	
	t = 0	t = 3	t = 0	t = 3	t = 0	t = 3
DCA content (%)	100	99.5	100	99.2	100	99.8
pH	6.8	5.02	6.75	5.04	4.8	4.75
Microbiological quality (after 1 month)	TAMC < 100 CFU/mL TYMC < 10 CFU/mL *E. coli* absent		TAMC < 100 CFU/mL TYMC < 10 CFU/mL *E. coli* absent		TAMC < 100 CFU/mL TYMC < 10 CFU/mL *E. coli* absent	

paediatric patients can be potentially affected by characteristics (i.e. taste, size, shape) of dosage forms. In this work, we have manufactured ODTs with specific characteristics. ODTs were round in shape, small in size and with a neutral/sweet taste.

Investigations of the API after molding process using DSC and HPLC analysis suggested that sodium DCA in the formulations was physically and chemically stable and no polymorphism occurred during the manufacture process. Based on these aspects, the molding technology proposed to produce sodium DCA ODTs could be a useful approach for hospital pharmacy or small laboratory that wants to manufacture an alternative dosage form for paediatric patients.

4. Conclusions

In this study we developed liquid and solid paediatric formulations of sodium DCA for the treatment of CLA, reserved for laboratory, such as a hospital pharmacy, and small-scale production, that could provide benefits over traditional dosage form. Preformulation studies on the active molecule were performed to identify those physico-chemical properties of the drug relevant to the design of the dosage forms and their process of manufacture. From the characterization of its solid-state and the investigation of its stability in aqueous solutions we were able to prepare three oral liquid formulations and manufacture orally disintegrating tables using molding technique. The formulations were manufactured with specific characteristics (i.e. taste, volume/size, shape, disintegration time) that, according to healthcare professionals opinions, could be more practicle and enhance acceptability and adherence of medication in these paediatric patients. Moreover, the screening and careful selection of excipients was a critical step in paediatric formulation development as certain excipients generally acceptable in children formulations, were not appropriate for children affected by CLA, e.g. saccharose in oral liquids formulations.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejps.2018.11.013.

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