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Folic acid in the solid-state: a synergistic computational, spectroscopic and structural approach

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Supporting Information Placeholder

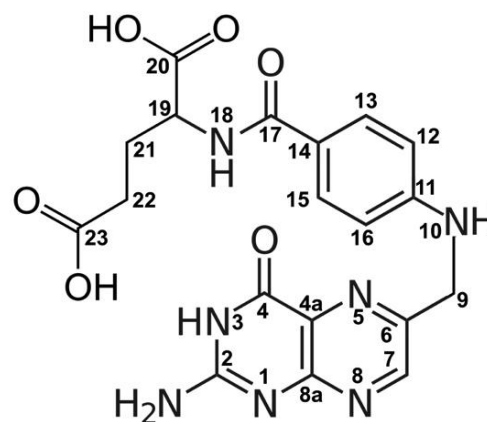
ABSTRACT: The structure of folic acid dihydrate has been investigated in the solid state by means of a synergistic approach combining Raman spectroscopy, X-ray powder diffraction and cutting-edge calculation methods. The comparison of the computed and measured Raman spectra was used to support the finding of a new crystalline form. Crystalline folic acid·2H₂O has also been used in the preparation, via solvent free methods, of amorphous multi-component materials and salts by reacting folic acid with LiOH, NaOH, Na₂CO₃ and Ca(OH)₂, which were also investigated by X-ray powder diffraction, thermogravimetric analysis, differential scanning calorimetry and intrinsic dissolution rate, and this has been compared with the values of the native vitamin. The preparation and characterization of the amorphous, hydrated adduct with LiCl is also reported.

INTRODUCTION

Folic acid ((2S)-2-[(4-[(2-amino-4-hydroxypteridin-6-yl) methyl]amino}phenyl)formamido]pentanedioic acid (Scheme 1), also known as vitamin B9, is the synthetic form of folate, which is used as a generic name to include the different forms of vitamin pteroylglutamates (with various levels of reduction of the pteridine ring). This vitamin and its biologically active derivatives act as coenzymes for the synthesis and metabolism of many amino acids and nucleotides by single carbon transfer reactions. This is a very important process in the metabolic pathways, and a folic acid deficiency can be associated to various diseases related to side effects such as neural tube defects (NTDs),¹⁻⁵ congenital heart defects and cardiovascular diseases.⁶ Vitamin B9 is not produced by the human body and must be taken through the diet in form of tablets. We reckoned that a deeper understanding of the structure

and solid state properties of this important molecule would foster new research and allow us to investigate co-crystallization and ionic co-crystals. The preparation and behaviour of polymorphs, amorphous forms, solvates and co-crystals/salts of an active pharmaceutical ingredient such as folic acid are attractive topics of research both at the academic and at the industrial level (pharmaceutical patenting as well as optimizing conditions for formulation).⁷⁻⁸ Furthermore, there is a strong interest in the preparation of new formulations of active pharmaceutical ingredients (APIs), in particular co-crystals, which often show physical and chemical properties (solubility, intrinsic dissolution rate, melting point, color, etc.) different from those of their separate components.⁹

For these reasons we have investigated the structure of folic acid in the solid state and the preparation and characterization of a series of new materials based on folic acid.



Scheme 1. Folic acid.

An X-ray structure of folic acid dihydrate was published in 1980.¹⁰ Unfortunately, the coordinates were not published, nor were they deposited in the Cambridge Structural Database.

With the intent of studying the relationships between structure and properties of folic acid in our quest for multiple crystal forms (co-crystals and salts),¹¹⁻¹³ we decided to redetermine the structure, but, in view of the difficulties experienced in obtaining good quality single crystals, we resolved to obtain a more detailed description of the solid-state structure of folic acid directly from powder diffraction data. However, folic acid is a flexible molecule, characterized by several degrees of freedom in the glutamic moiety, and structural determination from powder data is, for this reason, not straightforward. Indeed, we were able to obtain *two distinct solutions* for folic acid·2H₂O, differing only in the conformation of the glutamic moiety, and fitting almost equally the observed diffraction pattern. We called the first solution **FOL1** and the second **FOL2**. The solution **FOL2** pretty much resembled - as far as it could be judged from the stereo-view picture published as Figure 1 in ref. 10 - the conformation of the folic acid dihydrate previously reported. In the following we describe how we have managed to identify the correct structure of folic acid in our powder sample. This structure differs from the one previously reported; this fact might well hint the possibility of two conformational polymorphs¹⁴ for folic acid dihydrate.

EXPERIMENTAL SECTION

All reagents and solvents were purchased from Sigma-Aldrich and used without further purification.

Thermal gravimetric analysis (TGA). TGA measurements were performed using a Perkin-Elmer TGA7 in the temperature ranges 40-460 °C and 37-450 °C for the folic acid and the salts/co-crystal, respectively, under N₂ gas flow, at a heating rate of 5°C min⁻¹. The interaction between salt and folic acid is confirmed by the absence of the decomposition peak for folic acid in the TGA traces.

Differential scanning calorimetry (DSC). DSC measurements were performed with a Perkin-Elmer Diamond. Samples (3-10 mg) were placed in open aluminum pans. Heating was carried out at 5°C min⁻¹ for all the samples, in the temperature range 25-300°C.

Intrinsic dissolution rate (IDR). Dissolution rate in physiological solution at room temperature was measured for folic acid, the salts and the co-crystal. Measurements were carried out with a Varian Cary 50 Spectrophotometer equipped with a fiber optic dip probe. Four standard solutions in physiological solution (0.1 M NaCl) at concentrations 2.00, 4.00, 6.00, 8.00 mg L⁻¹ were used to calculate a calibration curve for all the samples (correlation coefficient was 0.9988). Absorbance of the solutions was measured, the linear part of the spectrum between 0.1 and 0.6 min utilized, and its slope calculated, corresponding to the dissolution rate in time interval, expressed in Abs min⁻¹. The Abs min⁻¹ values were then interpolated in the calibration curve to find the dissolution rate of the analytes expressed as g sec⁻¹.

X-ray powder diffraction (XRPD) Experiments. For phase identification purposes, X-ray powder diffractograms in the 2θ range 3-70° (step size 0.02°; time/step 20 s; 0.04° rad soller; VxA 40x40) were collected on a Panalytical X'Pert PRO automated diffractometer equipped with an X'Celerator detector in Bragg-Brentano geometry, using Cu Kα radiation without a monochromator.

For structure solution and refinement data a polycrystalline sample of folic acid was sealed in a 0.5 mm capillary, and an X-ray

powder diffractogram in the 2θ range 3-70° (step size 0.02°, time/step 15 s, VxA 40x40) was collected on a D8 Bruker diffractometer equipped with a primary Ge monochromator for Cu Kα1 and a Sol-X solid state detector in Debye-Scherrer geometry.

Variable temperature X-ray powder diffraction. X-ray powder diffractograms for folic acid·2H₂O were collected in the 2θ range 3-70° on a Panalytical X'Pert PRO automated diffractometer equipped with an X'Celerator detector and an Anton Paar TTK 450 system for measurements at controlled temperature. The data were collected in open air in Bragg-Brentano geometry using a Cu Kα radiation without a monochromator.

Structure determination from XRPD data. A volume of 2033.3(4) Å³ was found with the algorithm DICVOL.¹⁵ The asymmetric unit contains one molecule of folic acid and two water molecules. Space group determination with Highscore plus resulted in space group *P*2₁2₁2₁, with *Z* = 4, *Z'* = 1. The structure was solved by simulated annealing using all independent ions and molecules. Simulated annealing, that runs with structure fragments, was performed with EXPO2013,¹⁶ using one folic acid molecule and two independent water molecules. Ten runs for simulated annealing trial were set, and a cooling rate (defined as the ratio *T_n/T_{n-1}*) of 0.95 was used. Best solutions were chosen for Rietveld refinements, which were performed with the software TOPAS.¹⁷ A shifted Chebyshev function with 16 parameters and a Pseudo-Voigt function were used to fit background and peak shape, respectively. Soft constraints were applied for all bond distances and angles of the folic acid molecule, and a planar group restraint was applied to the aromatic ring. An overall thermal parameter was adopted for all atoms of folic acid. All the hydrogen atoms were fixed in calculated positions. Refinement converged with $\chi^2 = 1.28$ and *R_{wp}* = 9.68 (Table 2). The program Schakal99¹⁸ was used for all graphical representations.

Determination of unit cell parameters (Pawley refinement) of anhydrous folic acid at high temperature. Powder diffraction data collected at 423K were analyzed with the software Highscore plus. 15 peaks were chosen in the 2θ range 3-40°, and unit cell parameters were found thanks to the algorithm DICVOL. For the anhydrous form of folic acid a monoclinic unit cell with a volume of 3170.4(7) Å³ was found. The volume is consistent with the presence of three folic acid molecules in the asymmetric unit. The Pawley fit was performed with the software TOPAS. A shifted Chebyshev with 8 coefficients and a pseudo-Voigt function were used to fit background and peak shape, respectively. Refinement converged with $\chi^2 = 1.40$ and *R_{wp}* = 3.23 (Table 1).

Theoretical ab initio calculations.

The relative stability of the two crystalline structures of folic acid·2H₂O has been computed through the CRYSTAL¹⁹ ab initio quantum chemistry program. It is well known²⁰ that the treatment of molecular crystals is a delicate issue, due to the need to describe at the same qualitative level covalent and dispersive interactions. We used here a dispersion-corrected hybrid DFT functional (B3LYP) complemented with Grimme's empirical dispersion correction (more details in the Supporting Information). A 6-31G** basis was used for geometry optimizations and frequency calculations, while a cc-pVTZ basis set was adopted for the evaluation of relative stability. Raman spectra (vide infra) have been computed using the same computational setup, thanks to a novel algorithm recently implemented for the calculation of the intensity of vibrational bands.²¹

Raman spectroscopy. The Raman spectrum of folic acid dihydrate was recorded on a Bruker MultiRam FT-Raman spectrometer equipped with a cooled Ge-diode detector. The excitation source was an Nd³⁺-YAG laser (1064 nm) in the backscattering (180°) configuration. The focused laser beam diameter was about

100 μm and the spectral resolution 4 cm^{-1} . The spectrum was recorded with a laser power at the sample of about 40 mW.

Table 1. Details of data collection and structure refinement for FOL1, and Pawley fit of the anhydrous form obtained at HT.

| | Folic acid·2H ₂ O (FOL1) Structure refinement | Folic acid at HT (anhydrous form) Pawley fit |
|------------------|---|---|
| Formula | C ₁₉ H ₂₃ N ₇ O ₈ | C ₁₉ H ₁₉ N ₇ O ₆ |
| M _r | 477.40 | 336.25 |
| temperature/K | 293 | 423 |
| wavelength (Å) | 1.54056 | 1.54056 |
| crystal system | orthorhombic | monoclinic |
| space group | <i>P</i> 2 ₁ 2 ₁ 2 ₁ | <i>P</i> 2 ₁ |
| <i>a</i> /Å | 8.6163(9) | 28.812(3) |
| <i>b</i> /Å | 32.428(4) | 4.424(9) |
| <i>c</i> /Å | 7.2771(5) | 26.37(2) |
| β /deg | 90 | 109.38(2) |
| V/Å ³ | 2033.3(4) | 3170.4(7) |
| Z | 4 | 6 |
| Z' | 1 | 3 |
| Rwp | 9.68 | 3.23 |
| χ^2 | 1.28 | 1.40 |

[Values from ref. 4: space group *P*2₁2₁2₁, *a* = 7.295(2) Å, *b* = 8.655(3) Å, *c* = 32.545(15) Å, *V* = 2054.83 Å³; R1 (all data) = 0.184, R1 (observed) 0.146]

RESULTS AND DISCUSSION

The structure of folic acid dihydrate in the solid state

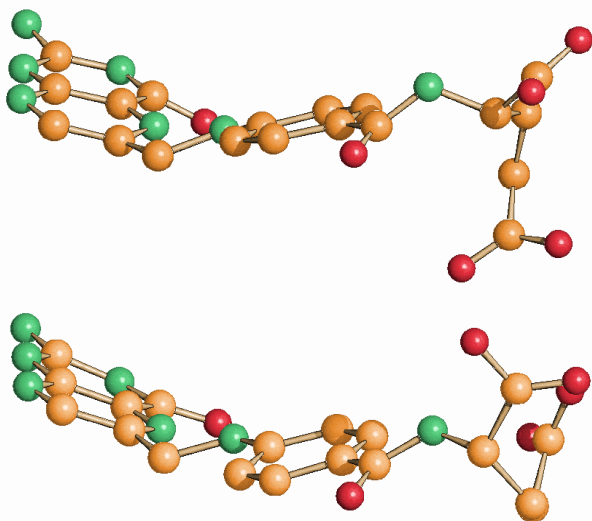


Figure 1. Different conformation of the glutamic moiety in the two solutions of folic acid·2H₂O from powder data: **FOL1** (top, new structural model) and **FOL2** (bottom, conformation similar to the one from ref. 10).

Structure solution via X-ray powder data on a crystalline sample of folic acid dihydrate allowed us to obtain two distinct solutions, differing mainly for the conformation of the glutamic moiety. The two different conformations, which we called **FOL1** and **FOL2**, are shown in Figure 1. **FOL1** (top) represents a new structural model, while **FOL2** (bottom) represents a conformation almost identical to the one reported in the 1980 article.¹⁰

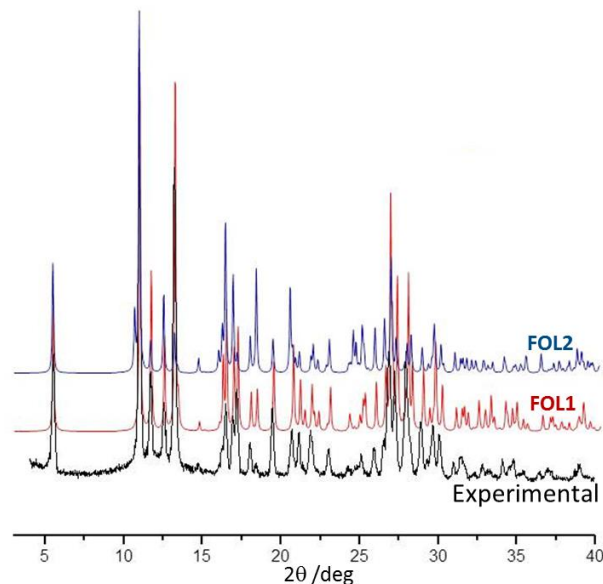


Figure 2. Comparison between the experimental diffraction pattern and those calculated on the basis of **FOL1** and **FOL2**.

Figure 2 shows a comparison between the experimental diffraction pattern and those calculated on the basis of **FOL1** and **FOL2**, respectively.

In order to tackle the ambiguity and attribute the correct conformation to the folic acid molecule, we decided to make use of complementary approaches: the correct structure corresponding to our powder sample could be assigned only through a combination of theoretical ab initio calculations and Raman spectroscopy measurements (see Experimental Section).

The crystal structure **FOL1** was found to be substantially more stable than the crystal structure **FOL2**, by an amount of 17.28 kJ/mol per unit formula (including vibrational contribution, see Table S1, Supporting Information). This, despite the fact that the isolated molecule itself is in a conformation that is, according to our calculations, less stable by about 10 kJ/mol (Table S1, Supporting Information). The latter result can be of interest in understanding the kinetics of the crystal formation and the relationship between optimization (minimization) of the crystal global free energy in the case of flexible molecules.

Vibrational Raman spectroscopy represents a powerful tool to investigate and identify substances because it provides fingerprint spectra that are unique to each specific compound and its molecular packing. Surface-enhanced Raman spectroscopic (SERS) studies in aqueous solutions have been aimed at setting up analytical methods to selectively detect folic acid in water and biological samples;²² moreover, resonance Raman studies in aqueous solutions have allowed to clarify the binding of folate and methotrex-

ate to dihydrofolate reductases.²³⁻²⁵ A recent Raman study on folic acid adsorbed on various metal substrates has been reported.²⁶ In the present investigation, the Raman spectrum of folic acid dihydrate was recorded and compared with the spectra obtained for **FOL1** and **FOL2** by theoretical calculations.

Figure 3 reports the simulated and measured spectra; the band wavenumber values and assignments were also compared (see Table S2, Supporting Information).

The spectrum *calculated* for **FOL1** shows a better agreement with the experimental data, especially in the low-wavenumber region, where lattice vibrations fall, and in the 1800 -1100 cm^{-1} range. The assignments obtained from theoretical calculations show a good agreement with those reported in the literature for folate and model compounds in solution^{23-25,27,28} as well as for solid model compounds^{25,29-32} and, more recently, for folic acid.²⁶ (see Table S2, Supporting Information). However, it is surprising that in the latter study no assignments to the pteridine ring stretching have been given; moreover, the low-wavenumber region has not been investigated.

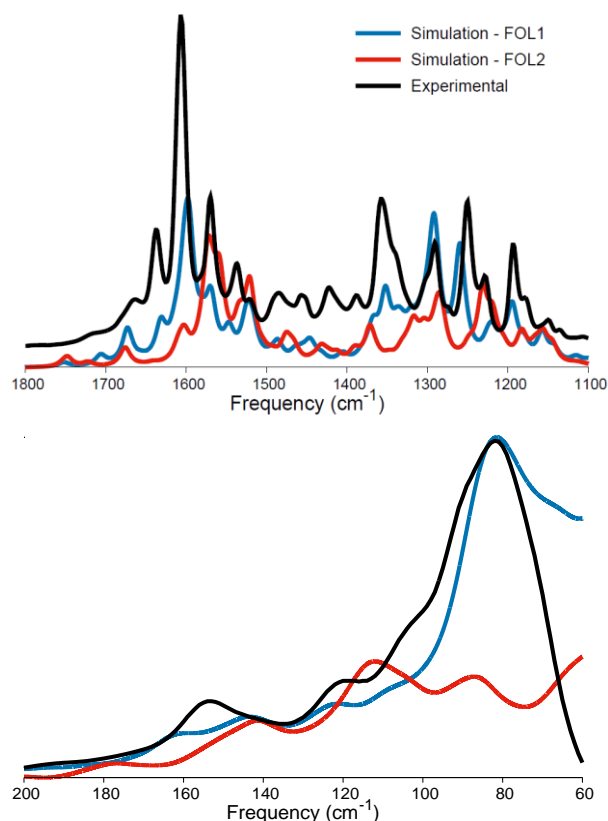


Figure 3. Experimental and calculated Raman spectra for folic acid·2H₂O at high (top) and low (bottom) frequencies.

The obtained results represent strong evidence supporting the proposed structure **FOL1** for the crystalline powder (see Figure 4). Each folic acid molecule is involved in a large number of hydrogen bonds with adjacent folic acid molecules: zig-zag chains of folic acid are arranged in corrugated sheets, interspersed with bridging water molecules, resulting in the pattern shown in Figure 5. Figure 5 (bottom) shows how the two independent water molecules, though organized in channel-like fashion, are closely interacting with the hydrophilic part of the organic framework; for this

reason they do not easily leave the crystal, and the dehydration process is observed only at high temperature, between 100 and 150°C (see below for TGA measurements).

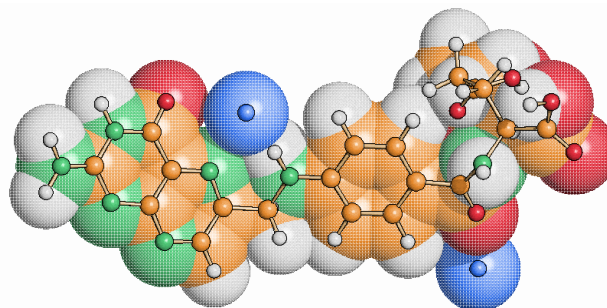


Figure 4. The molecular structure of **FOL1** in the solid state. The refinement is based on the model **FOL1** derived from the Raman and computational experiments.

Variable temperature X-ray powder diffraction experiments on the dihydrate form have been carried out to verify the crystal structure stability after the dehydration process (Figure 6). Loss of water and subsequent solid-solid transition to the anhydrous phase have been observed at 150°C. After cooling back to 25°C, restoration of the hydrated phase occurred in a few minutes; increase in the crystallinity of folic acid could be observed upon time (Figure 6). Therefore, the dihydrated crystal undergoes a reversible water loss/water uptake process in between crystalline phases. All attempts to determine the structure of the high temperature (HT) form from powder diffraction data have so far been unsuccessful, due to the poor quality of the diffraction pattern. However, we have been able to obtain unit cell parameters and space group (see Table 2 and the Experimental section); Figure 7 shows the experimental, calculated and difference diffraction patterns.

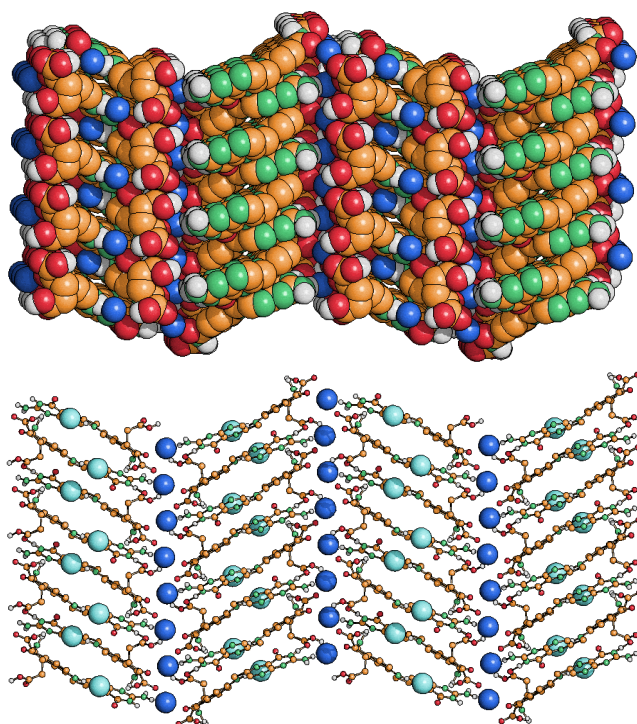


Figure 5. Top: Sheets of folic acid molecules in crystals of folic acid·2H₂O (**FOL1**). Bottom: The two independent water molecules are indicated here in blue and light-blue, respectively; note how the *blue* ones occupy channels in the crystal.

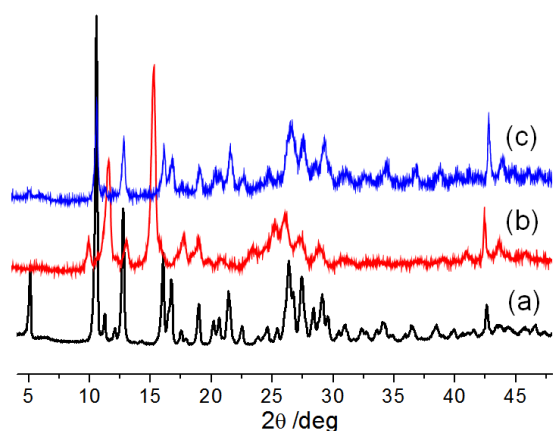


Figure 6. Variable temperature XRPD measurements on a crystalline sample of folic acid·2H₂O: at room temperature (a), at 150°C on heating (b) and back at room temperature (c).

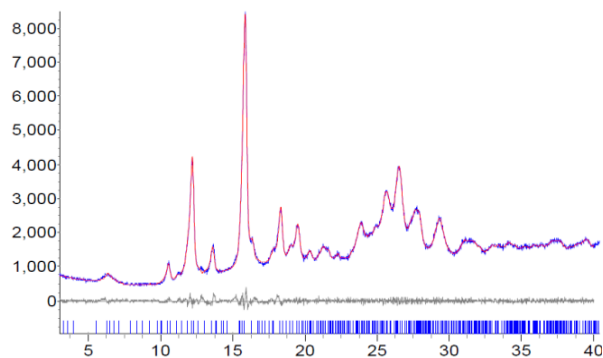


Figure 7. Experimental (blue), calculated (red), and difference (grey) powder patterns for HT folic acid. Peak positions are marked in blue.

Hydrated salts and co-crystals of folic acid

The solid-state characterization of folic acid was part of a more general study on the solid-state reactivity of folic acid towards inorganic bases and salts, in order to explore the possible formation of salts or ionic co-crystals, these latter by using inorganic salts as co-formers with solvent free methods (see Experimental). Hydrated salts of folic acid with NaOH and Ca(OH)₂ were obtained by manually kneading together folic acid·2H₂O in 1:2 and 1:1 stoichiometric ratio, respectively, in an agate mortar for 20 min with a drop of water; all reactions were quantitative. Hydrated lithium folate and a hydrated 1:2 folic acid adduct with LiCl were obtained by ball milling for 30 min (using a Retsch MM200 grinder mill operated at a frequency of 20 Hz) folic acid with LiOH and LiCl, respectively, in a 1:2 molar ratio, with the addition of a few tiny drops of water; the reactions were quantitative. An analogous solid-state test with Na₂CO₃ resulted in the formation of the hydrated sodium folate, with production of CO₂ and water. Solid-state reactivity of folic acid with the inorganic salts NaCl, KCl and CaCl₂ was also tested by manual grinding/kneading and ball milling, but the solid obtained was in all cases a physical mixture of the reagents. All products were analyzed by XRPD and the diffraction powder patterns were invariably those characteristic of an amorphous phase. The diffraction patterns in the case of lithi-

um salts are shown in Figure 8 as an example: both the lithium salt and the amorphous adduct folic acid·LiCl show an amorphous pattern. Amorphous drugs are a desired target, when solubility issues are at stake, as they usually show a better dissolution rate with respect to crystalline material.³³ At the same time, given that an amorphous material represents a metastable phase, it is usually important to check for behavior with time; in all the cases presented here, amorphicity could still be observed after approximately 12 months. Even in the case of the amorphous adduct folic acid·LiCl the X-ray powder pattern does not show traces of residual reagents, i.e. the interaction between the components is at molecular level. This is confirmed via TGA/DSC measurements. TGA measurements (see Supporting information) were used to calculate the stoichiometric water contents.

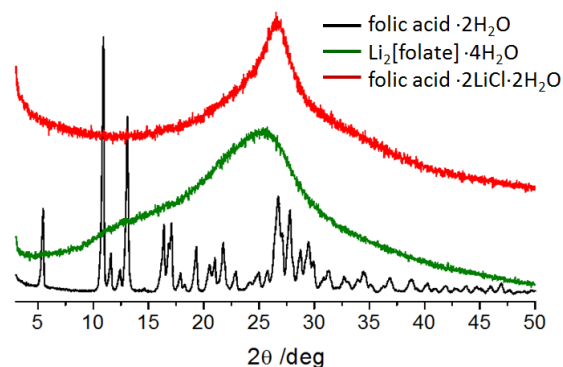


Figure 8. Comparison of XRPD patterns for folic acid dihydrate and the products of reactions with LiOH (green line) and LiCl (red line).

Figure 9 shows a comparison of the TGA traces for pure folic acid·2H₂O (top) and Li₂[folate]·4H₂O (bottom), respectively. The salt decomposition occurs at about 350 °C (i.e. the folate in its salts is 100°C more stable than pure folic acid),³⁴ residual traces of unreacted folic acid can be detected as a small change in the slope at about 250°C, corresponding to folic acid melting and decomposition.

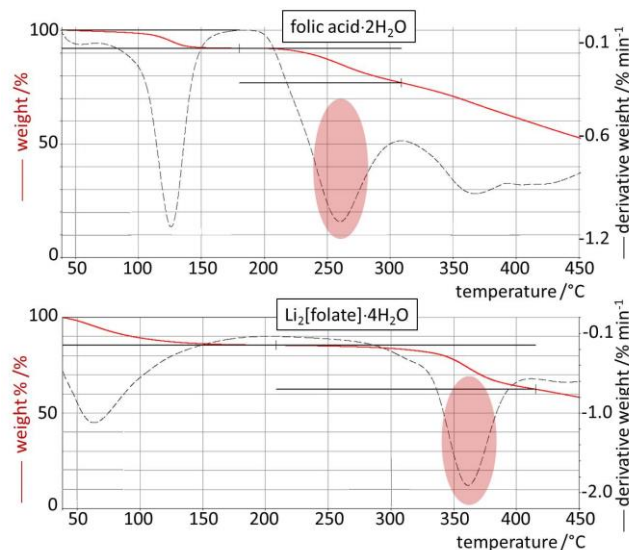


Figure 9. Thermogravimetric analysis of folic acid·2H₂O (top) and Li₂[folate]·4H₂O (bottom). Decomposition temperatures are evidenced by the large pink ovals.

Intrinsic dissolution rate (IDR)

Measurements in physiologic saline solution show an increase, with respect to pure folic acid,³⁵ of IDR values for all compounds, as it is shown in Figure 10 and in Table 2.

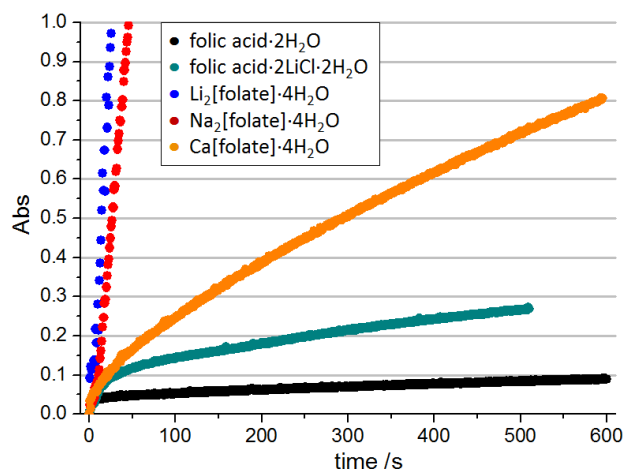


Figure 10. Intrinsic dissolution rate (IDR) measurements.

Table 2. Intrinsic dissolution rates

| Solid compound | $\text{g s}^{-1} (\times 10^5)$ |
|--|---------------------------------|
| folic acid·2H ₂ O | 1.27 |
| folic acid·2LiCl·2H ₂ O | 1.59 |
| Ca[folate]·4H ₂ O | 1.67 |
| Na ₂ [folate]·4H ₂ O | 4.96 |
| Li ₂ [folate]·4H ₂ O | 8.62 |

A clear trend can be observed: intrinsic dissolution rate increases from pure acid to the LiCl adduct, in which the folic acid is still in its molecular form. The increase is definitely larger for all the folate salts, with a lower value for the divalent calcium cation; the largest value is observed for lithium, probably due to the large difference in size between anion and cation, and to the higher hydration enthalpy expected for the lithium cation with respect to sodium.

CONCLUSION

In this paper the solid-state structure of folic acid dihydrate has been redetermined from powder diffraction data. In view of the structural non rigidity of folic acid, the structure optimization has been guided by the convergent and complementary use of information derived from Raman spectroscopy and computational methods, which allowed to discriminate between two structural models **FOL1** and **FOL2**, differing in the conformation of the glutamic moiety. The crystal structure of folic acid dihydrate has been used, in turn, to rationalize the de-hydration process, although it has not been possible to fully characterize the HT phase. Our results demonstrate that the combination of different techniques applied to the solid state is necessary to tackle complicated structural problems. As the structure corresponding to the FOL2 solution is similar to the structure previously reported,¹⁰ it is likely that the two forms are conformational, monotropically related polymorphs.

Folic acid dihydrate has also been reacted by solvent free methods with LiOH, NaOH, and Na₂CO₃ and Ca(OH)₂ and treated with LiCl, NaCl, KCl and CaCl₂ to explore the formation of the folate salts and of the amorphous adduct of LiCl with folic acid. All compounds were characterized by XRPD, TGA/DSC, and their intrinsic dissolution rate was measured. Very stable amorphous phases were obtained, including the interesting amorphous folic acid-LiCl adduct. This study highlights the importance of using different routes of preparation to obtain new formulation of APIs. In particular, the solid-solid reaction can be used to produce new forms of a same API with different physical chemical parameters.

ASSOCIATED CONTENT

Supporting information

The Supporting Information is available free of charge on the [ACS Publications website](http://pubs.acs.org) at DOI:xx.xxx/acs.cgd.xxxxxxx: Raman spectra and assignments, computational details and TGA traces. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Accession Codes

CCDC 1446314 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif, or by emailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge CB2 1EZ, UK; fax: +44 1223 336033.

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Notes

The authors declare no competing financial interests.

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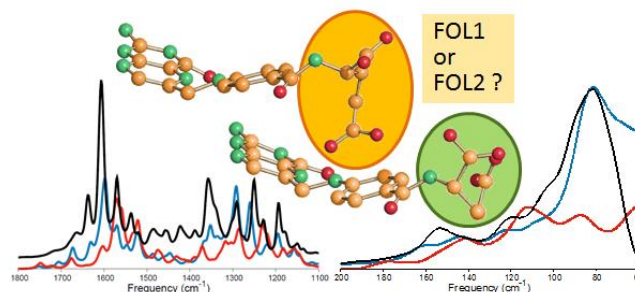
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Folic acid in the solid-state: a synergistic computational, spectroscopic and structural approach

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Synopsis: The structure of crystalline folic acid·2H₂O has been investigated by a synergistic approach combining Raman spectroscopy, X-ray powder diffraction and cutting-edge calculation methods. The comparison of the computed and measured Raman spectra was used to support the proposed solid-state structure. Folic acid·2H₂O has also been used in the preparation, via solvent free methods, of amorphous multi-component materials and salts.

