

Journal of Zhejiang University-SCIENCE B (Biomedicine & Biotechnology) 2022 23(7):597-606 www.jzus.zju.edu.cn; www.springer.com/journal/11585 E-mail: jzus_b@zju.edu.cn

Correspondence https://doi.org/10.1631/jzus.B2100970

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Detection, isolation, and characterization of a novel impurity from several folic acid products

Qirong SHEN^{1,3*}, Quan HE^{1*}, Yuanjiang PAN², Cuirong SUN^{1⊠}

¹College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China ²Department of Chemistry, Zhejiang University, Hangzhou 310027, China ³Hangzhou Qianyuan Pollen Pharmaceutical Co., Ltd., Hangzhou 310018, China

Folic acid belongs to the group of water-soluble B vitamins and naturally exists in multiple forms in a wide variety of foods such as legumes, vegetables, liver, and milk (Iyer and Tomar, 2009; Lyon et al., 2020). It is involved in many biochemical reactions critical for cell division, such as purine and pyrimidine biosynthesis, DNA/RNA biosynthesis, and amino acid metabolism (Iver and Tomar, 2009). Mammals cannot synthesize folic acid and thus they must acquire it from food. Although folic acid is ubiquitous in foods, folic acid deficiency still often occurs due to various causes such as unhealthy diet (Hildebrand et al., 2021; Iimura et al., 2022), disease-related malabsorption (Arcot and Shrestha, 2005), medication-related depletion (Arcot and Shrestha, 2005), or vitamin B12 deficiency (Fishman et al., 2000). Folic acid deficiency has been associated with several health problems, such as anemia (Carmel, 2005; Bailey and Caudill, 2012), cancer (Duthie, 1999), cardiovascular diseases (Wald et al., 2002), neural tube defects in newborns (van der Put et al., 2001), neuropsychiatric dysfunction (Shea et al., 2002), depression (Falade et al., 2021), inflammatory diseases (Suzuki and Kunisawa, 2015; Jones et al., 2019), and eye diseases (Sijilmassi, 2019). To prevent folic acid deficiency, its daily intake (400 μ g/d) has been recommended for adults in the European Union, and its increased intake (600 μ g/d) is advised for women before and during pregnancy (FAO/WHO, 2002; IOM, 2004). The New Zealand government mandated the fortification of non-organic wheat flour with folic acid in July 2021, and the UK government mandated the fortification of non-wholemeal wheat flour with folic acid in September 2021 (Haggarty, 2021).

Structurally, folic acid is a conjugated pterin derivative consisting of three moieties: a 6-methylpterin residue, a *p*-aminobenzoic acid residue, and a glutamic acid residue (Fig. 1). Folic acid can be artificially synthesized using either a single-step process or a twostep process (Kłaczkow and Anuszewska, 2006). The single-step process is a condensation reaction of an appropriate pyrimidine with an alkaline three-carbon molecule and N-4-aminobenzoyl-L-glutamic acid. In the two-step process, the pterin part is synthesized first and then the synthesized pterin species condenses with N-4-aminobenzoyl-L-glutamic acid to yield the folic acid. Folic acid is inexpensive to manufacture, more stable during processing and storage than most members of the folate family, and can be efficiently metabolized in vivo into biologically active derivatives such as 5-methyltetrahydrofolic acid. Due to these advantages, manufactured folic acid is often used in a tablet form and in fortified foods for nutrition supplementation (Lucock, 2000).

During the steps of pharmaceutical synthesis, processing, and storage, impurities are inevitably produced, which may cause potential toxicity when drugs are taken by patients. In this respect, the Viracept accident is a good example (Müller and Singer, 2009). Viracept is a human immunodeficiency virus (HIV) protease inhibitor, first introduced by Roche in 1998. On May 18, 2007, Roche received reports regarding the "bad smell" of some Viracept products and symptoms

Cuirong SUN, suncuirong@zju.edu.cn

^{*} The two authors contributed equally to this work

D Cuirong SUN, https://orcid.org/0000-0002-4479-8013

Received Nov. 25, 2021; Revision accepted Mar. 14, 2022; Crosschecked May 13, 2022

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Fig. 1 Chemical structure of folic acid.

of nausea and vomiting in Spanish patients taking the medicine. Subsequently, ethyl mesylate, an established genotoxic impurity, was identified in the Viracept products as the source of the bad smell and symptoms. It turned out that ethyl mesylate was formed in an excess amount due to an undesirable reaction between the starting material and the solvent in the production tank. On June 6, 2007, all related Viracept products had to be globally recalled. The Viracept incident clearly demonstrates the importance of screening for impurities in active pharmaceutical ingredients (APIs) and limiting the amounts thereof in the final products.

To date, a number of impurities, including starting materials, synthetic intermediates and byproducts, degradation products, etc., have been identified in folic acid products (Dántola et al., 2010, 2018; The European Pharmacopoeia Commission, 2018; Pharmaffiliates Analytics and Synthetics P. Ltd., 2021). The European Pharmacopoeia 9.5 includes eight registered impurities (Fig. 2; impurities A-H) (The European Pharmacopoeia Commission, 2018). The photooxidation of folic acid yields two further impurities, 6formylpterin (Fop) and 6-carboxypterin (Cap) (Fig. 2) (Dántola et al., 2010, 2018), which are not recorded in pharmacopoeias. Other possible non-pharmacopeial impurities include 4-aminobenzoic acid, L-glutamine, etc. (Pharmaffiliates Analytics and Synthetics P. Ltd., 2021). All of the reported impurities are structural analogues of folic acid. In this work, a novel impurity, 4-((2-amino-7-methyl-4-oxo-1,4-dihydropteridin-6-yl) amino)benzoic acid (hereafter termed as "impurity K"), was detected in several commercial folic acid products and successfully isolated from one of them. Its chemical structure was characterized using multidimensional nuclear magnetic resonance (NMR) in combination with Fourier transform-infrared (FT-IR) spectroscopy, ultraviolet-visible (UV-Vis) absorption spectroscopy,

and quadrupole time-of-flight high-resolution mass spectrometry (QTOF-HRMS) techniques.

First, a commercial folic acid product was analyzed with high-performance liquid chromatography (HPLC) (Fig. 3a), and most peaks observed were attributed to known impurities such as impurities A, D, G, H, and Fop, as well as other non-pharmacopeial structural analogs (peaks 1-7) of folic acid (Table S1). The retention time (RT) of folic acid and all of the detected impurities has been summarized in Table S2. Besides these known impurities, a novel impurity, i.e., impurity K, was detected at a level of less than 0.04% based on the HPLC peak area (Fig. 3a). This impurity was also detected in several other folic acid products (Fig. S1), suggesting that impurity K should be a general impurity. The RT of impurity K was observed to be (20.325±0.045) min, and its relative RT reference to folic acid (RT=(4.917±0.090) min) was determined to be 4.13. Subsequently, impurity K was isolated from one folic acid product using preparative HPLC. The purity of the isolated impurity K sample was determined using analytical HPLC as (99.47±0.06)%, which is pure enough for structural analysis (Fig. 3b). The isolated impurity K sample is a yellow-brown powder in appearance.

Second, the chemical structure of impurity K was established. Several techniques have been successfully applied in this regard, including FT-IR, UV-Vis, HRMS, and multidimensional NMR, in combination with the NMR/UV-Vis data of folic acid (Rossi et al., 1992) as well as the impurities D and G. Herein, the QTOF-HRMS software predicted the most likely molecular formula of impurity K (Fig. S2) to be $C_{14}H_{12}N_6O_3$ (measured [M+H]⁺ mass-to-charge ratio (*m/z*) 313.1041 vs. theoretical [M+H]⁺ *m/z* 313.1044; Fig. 4a), which is consistent with the ¹H- and ¹³C-NMR results (see below). This molecular formula indicated that the degree of unsaturation is 12, suggesting the presence



Fig. 2 Chemical structures of ten main impurities in folic acid products.

of heteroatom-containing aromatic structure. In addition, the $[M+K]^+$ ion at m/z 351 could also be observed. The tandem mass spectrometry (MS/MS) analysis showed that the protonated impurity K loses one H₂O molecule (18 Da) and then further loses one CO molecule (28 Da) during the fragmentation, yielding the peaks at m/z 295 and 267 (Fig. 4b), respectively, suggesting that impurity K probably contains a carboxylic group. The ion at m/z 267 could further lose one NH₃ group to yield the peak at m/z 250 (Fig. 4b), suggesting the presence of an $-NH_2$ group.

The FT-IR analysis (Fig. 4c) suggested the presence of several characteristic chemical groups in impurity K, including NH₂ (3448, 3321 cm⁻¹), COOH (broad band, 3118 cm⁻¹), aliphatic C–H (2844, 1464, 1380 cm⁻¹), aromatic C–H (857 cm⁻¹), aromatic C–C (1601, 1508, 1415 cm⁻¹), and C=O (1685 cm⁻¹). The detection of NH₂, COOH, and aromatic bands is consistent with the MS and MS/MS results. The aromatic C–C band at 1415 cm⁻¹ further indicated the presence of heteroatom-containing aromatic structure. Furthermore, the bands at 1464 and 1380 cm⁻¹ suggested the presence of methyl group in impurity K.

The UV-Vis spectroscopy analyses of impurity K (Fig. 4d) and folic acid (Fig. 4e) indicated that both molecules show an absorption maximum at the



Fig. 3 High-performance liquid chromatography (HPLC) chromatograms of the commercial folic acid product (a) and the isolated impurity K sample (b). The ultraviolet-visible (UV-Vis) absorption spectroscopy wavelength is 317 nm. Fop: 6-formylpterin; Cap: 6-carboxypterin; mAU: milli-absorbance unit.

wavelength range of 250–350 nm, also suggesting the presence of an aromatic structure. Compared with folic acid, impurity K shows a redshift of the absorption maximum from 282 nm to 316 nm, suggesting that an auxochrome probably links directly to the aromatic ring system.

The ¹H-NMR analysis suggested the presence of 12 hydrogens in the compound (Fig. 5a), which is consistent with the molecular formula predicted by the MS analysis. The signals at $\delta_{\rm H}$ 2.50 and 3.34 represent traces of dimethyl sulfoxide (DMSO) and H₂O, respectively (Fig. 5a). The large signal at $\delta_{\rm c}$ 39.39 also represents trace DMSO (Fig. 5b). The signals at $\delta_{\rm H}$ 2.62 and $\delta_{\rm c}$ 21.69 indicate the presence of methyl group in impurity K (Fig. 5), consistent with the FT-IR results. The signals at $\delta_{\rm c}$ 167.04 and 160.73 indicate the presence of two carbonyl groups that may link to heteroatoms (N and O) (Fig. 5b). The signals at δ_c 151.90, 151.56, 150.74, 145.39, 144.76, 130.04, 122.53, 122.34, and 117.34 probably represent aromatic carbons in impurity K (Fig. 5b).

The ¹³C-NMR spectrum shows 12 carbon signals corresponding to 14 carbons of impurity K (Fig. 5b), suggesting the presence of some structural symmetry and two pairs of chemically equivalent carbons. The positive signals at δ_c 130.04 and 117.34, shown in the distortionless enhancement by polarization transfer (DEPT)-135 spectrum (Fig. S3), further indicate that the corresponding carbons should be in an aromatic CH group, which correlates with the proton signals at $\delta_{\rm H}$ 7.98 (2H) and 7.86 (2H) (Figs. 5a and S4), indicating the presence of a para-substituted benzene ring. Taken together, it was inferred that there are two pairs of aromatic CH groups in impurity K.



Fig. 4 Electrospray ionization mass spectrometry (ESI-MS) (a), tandem mass spectrometry (MS/MS) (b), Fourier transform-infrared (FT-IR) (c), and ultraviolet-visible (UV-Vis) absorption (d) spectra of impurity K, and the UV-Vis (e) spectrum of folic acid. cps: counts per second; mAU: milli-absorbance unit.

Fig. 5 ¹H-NMR (a) and ¹³C-NMR (b) spectra of impurity K. NMR: nuclear magnetic resonance; ppm: part per million.

In addition, the positive signal at $\delta_c 21.69$ further confirms the presence of a methyl group (Fig. S3), and no negative signals were observed in the DEPT-135 spectrum, indicating the lack of CH₂ groups in impurity K. The ¹³C-NMR signals at δ_c 167.04, 160.74, 151.90, 151.56, 150.74, 145.39, 144.76, 122.53, and 122.34 disappear in the DEPT-135 spectrum, suggesting that the corresponding carbons (nine carbons) have no protons attached.

Two pairs of aromatic CH groups and one methyl group account for seven hydrogens linking to carbon. Thus, the five hydrogens left should link to other atoms (N and O), and their proton signals are at $\delta_{\rm H}$ 6.50 (2H), 8.71 (1H), 11.30 (1H), and 12.48 (1H) (Fig. 5a). These protons should be exchangeable, which was confirmed by the hydrogen/deuterium (H/D) exchange experiment (Fig. S4). After D₂O was added, the peaks at $\delta_{\rm H}$ 6.5, 8.7, 11.3, and 12.5 all disappeared. Moreover, we inferred that the signals at $\delta_{\rm H}$ 6.50 and 12.48 should represent the NH₂ and COOH groups, respectively.

Like other impurities, impurity K probably has some structural similarity to folic acid. Thus, we examined the structure of folic acid considering the above inferred structural information, and found that, if the glutamic acid residue is hydrolytically removed from folic acid, the hydrolysis product, i.e., impurity D (Fig. 2), will comply with almost all of the above inferences: its molecular formula is $C_{14}H_{12}N_6O_3$, it has 12 unsaturations with two carbonyl groups, it has two pairs of chemically equivalent carbons (C-12/16 and C-13/15), and it has one $-NH_2$ group.

However, this hydrolysis product (impurity D) has a methylene group rather than a methyl group, while impurity K has methyl group instead of a methylene group, as indicated by the FT-IR and DEPT-135 NMR measurements. We subsequently found that one isomer of the impurity D, i.e., Structure 1 shown in Fig. 6, matches all of the experimental observations, including the methyl group, the redshift of the absorption maximum observed in the UV-Vis spectra (Fig. 4d), the correlations observed in the heteronuclear single quantum coherence (HSQC), ¹H-¹H correlation spectroscopy (COSY), and heteronuclear multiple-bond correlation (HMBC) spectra (Figs. S5-S7), etc. We also examined other possible isomers of impurity D, but it turned out that they all show some levels of structural contradiction with at least one of the aforementioned experimental observations.

Fig. 6 Chemical structure of impurity K.

Structure 1 has nine carbons with no proton attached and five proton-attached carbons. The direct linkage of an auxochrome group (–NH–) to the pterin moiety successfully explains the redshift of the UV absorption maximum, as compared with folic acid. The HSQC spectrum clearly indicated the one-bond correlations at H-9/C-9, H-12/C-12, H-13/C-13, H-15/C-15, and H-16/C-16 (Fig. S4). The ¹H-¹H COSY data revealed a three-bond correlation between H-12 and H-13, and that between H-15 and H-16 (Fig. S6).

The HMBC spectrum revealed multiple-bond correlations between a variety of hydrogens (H-9, H-10, H-12/16, and H-13/15) and carbons (C-4a, C-6, C-7, C-11, C-12/16, C-13/15, C-14, and C-17) (Fig. S7). Specifically, the correlations of H-10/C-4a and H-9/ C-4a were observed, which could probably be attributed to the planar zig-zag (W coupling) configuration (Araya-Maturana et al., 2008; Claridge, 2008). Both correlations are quite weak due to their long-range feature (four-bond for H-10/C-4a and five-bond for H-9/C-4a). The H-9/C-8a correlation was not observed due to the lack of the planar zig-zag configuration. In addition, the H-10/C-7 correlation is strong while the H-10/C-6 correlation was not observed, and the H-9/C-6 correlation is stronger than the H-9/C-7 correlation, indicating that the correlations of H-10/C-7 and H-9/C-6 should be three-bond correlations and the H-9/C-7 correlation should be a two-bond correlation. Taken together, these correlations clearly demonstrated that N-10 directly links to C-6, and C-9 directly links to C-7 in Structure 1. Moreover, the chemical shift of C-6 (150.74) is upfield compared to that of C-7 (144.76), further supporting that C-6 is connected to the electronegative N-10.

An interesting observation concerns the exchangeable protons of the amine groups. The 2-NH₂ ($\delta_{\rm H}$ 6.50) and H-1 ($\delta_{\rm H}$ 11.30) protons in Structure 1 showed a broad ¹H-NMR peak while the H-10 ($\delta_{\rm H}$ 8.71) proton showed a quite sharp peak (Fig. 5a). Also, the 2-NH₂ and H-1 protons showed no correlations with nearby carbons, while the H-10 proton showed correlations with the C-12/16, C-4a, and C-7 carbons in the HMBC spectrum (Fig. S7). It is possible that the exchange rate of the H-10 proton is much slower than those of the 2-NH₂ and H-1 protons, thus resulting in the sharp peak and the observed correlations.

The H-1 proton of Structure 1 deserves further attention. This proton can be delocalized to either N-3 or O-4 to afford further two resonance structures (Structures 2 and 3 in Fig. S8). Thus, it is quite critical to identify where this proton is exactly located in impurity K. According to the European Pharmacopoeia, it should be located at N-1 for folic acid and all of the registered impurities. Thus, we used folic acid (Fig. 1) and the impurities D and G (Fig. 2) as standard, and compared the chemical shifts of this proton in these compounds. We speculated that, if the chemical shift of this proton for impurity K is the same or similar to those for folic acid, impurities D and G, and then this proton should also be located at the N-1 position of impurity K. Wen (2017) has reported the chemical shift of this proton for folic acid to be 11.40. The chemical shifts for impurities D and G were found to be 11.50 and 11.89 (Fig. S9), respectively, and the chemical shift for impurity K was found to be 11.30 (Fig. 5a). These data clearly indicated that the position of this proton for impurity K should be the same as those for folic acid and the impurities D and G, i.e., it is at the N-1 position.

The ¹H and ¹³C chemical shifts at most positions of Structure 1 are similar to those of folic acid (Table S3), demonstrating the structural similarity between these two compounds. Meanwhile, there are some dissimilarities. The chemical shift of H-12/16 is upfield than that of H-13/15 in the case of folic acid, while the contrary is true for the case of Structure 1. The chemical shifts of H-9 and H-10 considerably change from 4.59 and 7.02 in folic acid to 2.62 and 8.71 in Structure 1, respectively. The chemical shift of C-9 greatly changes from 45.92 in folic acid to 21.69 in Structure 1. These chemical shift variations are probably due to the migration of C-9 from C-6 to C-7 and the direct linkage of N-10 to C-6, which change the electron density distribution around H-9, H-10, H-12/16, H-13/15, and C-9.

Altogether, Structure 1 was demonstrated to be the structure of the impurity we detected in several folic acid products, which has been termed as "impurity K." This impurity is a structural isomer of impurity D that is the hydrolysis product of folic acid (Fig. 2). All chemical shift and correlation data of impurity K are listed in Table 1. We compared the structures between impurity K and other folic acid impurities, and found that impurity K shows a greater structural similarity to impurity G (Fig. 2). This is also supported by the comparison of the UV-Vis (Fig. S10) and ¹H-NMR (Fig. S9) spectra of impurities K, D, and G. The UV-Vis and ¹H-NMR spectra of impurity K are more similar to those of impurity G than to those of impurity D. It is likely that impurity K is the hydrolysis product of impurity G, given that the latter is the most abundant impurity in most of the tested folic acid products (Figs. 3a and S1). This newly discovered impurity has not been recorded in pharmacopoeias.

In summary, the International Union of Pure and Applied Chemistry (IUPAC) name and the physicochemical properties of impurity K are listed as follows: 4-((2-amino-7-methyl-4-oxo-1,4-dihydropteridin-6-yl)

Position No.	$^{1}\mathrm{H}\left(\delta_{_{\mathrm{H}}}\right)$	$^{13}\mathrm{C}\left(\delta_{\mathrm{c}}\right)$	DEPT-135	HSQC	¹ H- ¹ H COSY	HMBC
1	11.30 (NH) (1H, s)					
2	6.50 (NH ₂) (2H, s)	151.90	С			
3						
4		160.73	С			
4a		122.34	С			
5						
6		150.74	С			
7		144.76	С			
8						
8a		151.56	С			
9	2.62 (3H, s)	21.69	CH_3	C-9		C-6, C-7, C-4a
10	8.71 (NH) (1H, s)					C-4a, C-7, C-12, C-16
11		145.39	С			
12	7.98 (1H, d)	117.34	СН	C-12	H-13	C-11, C-13, C-14, C-16
13	7.86 (1H, d)	130.04	СН	C-13	H-12	C-11, C-12, C-15, C-17
14		122.53	С			
15	7.86 (1H, d)	130.04	СН	C-15	H-16	C-11, C-13, C-16, C-17
16	7.98 (1H, d)	117.34	СН	C-16	H-15	C-11, C-12, C-14, C-15
17	12.48 (OH) (1H, s)	167.04	С			

 Table 1 NMR data summary of impurity K

NMR: nuclear magnetic resonance; DEPT-135: distortionless enhancement by polarization transfer-135; HSQC: heteronuclear single quantum coherence; COSY: correlation spectroscopy; HMBC: heteronuclear multiple-bond correlation.

amino)benzoic acid; yellow-brown powder; UV-Vis λ (1/3, 100 mmol/L CH₃COONH₄ (pH 5.8)/CH₃OH, volume ratio) (nm): 200, 262, 316 (λ_{max}); IR (KBr) v_{max} (cm^{-1}) : 3448 (s, sh), 3321 (s, sh), 3118 (vs, br), 2844 (s, sh), 1685 (s), 1601 (s), 1530 (s, sh), 1508 (s), 1464 (*m*, *sh*), 1415 (*s*), 1380 (*s*, *sh*), 1353 (*m*, *sh*), 1320 (*m*), 1288 (s), 1247 (s), 1176 (s), 1122 (w, sh), 1001 (w), 928 (w), 857 (m); ¹H-NMR (500 MHz; DMSO-*d*₆; tetramethylsilane (TMS)) (ppm): $\delta_{\rm H}$ 2.62 (3H, s, H-9), 6.50 (2H, s, 2-NH₂), 7.86 (1H, d, H-13), 7.86 (1H, d, H-15), 7.98 (1H, d, H-12), 7.98 (1H, d, H-16), 8.71 (1H, s, H-10), 11.30 (1H, s, H-1), 12.48 (1H, s, 17-OH); ¹³C-NMR (125 MHz; DMSO- d_6 ; TMS) (ppm): $\delta_{\rm C}$ 21.69 (C-9), 117.34 (C-12/16), 122.34 (C-4a), 122.53 (C-14), 130.04 (C-13/15), 144.76 (C-7), 145.39 (C-11), 150.74 (C-6), 151.56 (C-8a), 151.90 (C-2), 160.73 (C-4), 167.04 (C-17); ms ([M+H]⁺) (m/z): 313.1041 (Calc. 313.1044); ms ($[M+K]^+$) (m/z): 351.0607 (Calc. 351.0603); ms ($[M+H-H_2O]^+$) (m/z): 295.0941 (Calc. 295.0938); ms ($[M+H-H_2O-CO]^+$) (m/z): 267.0996 (Calc. 267.0989); ms ($[M+H-H_2O-CO-NH_3]^+$) (m/z): 250.0734 (Calc. 250.0723).

Materials and methods

Detailed information about experimental materials and methods could be found in the electronic supporting information of this paper.

Acknowledgments

This work was supported by the National Basic Research Program (973) of China (No. 2016YFF0200503) and the National Natural Science Foundation of China (No. 21876146).

Author contributions

Qirong SHEN performed the experimental research and data analysis, and wrote the original manuscript. Quan HE performed the experimental research and data analysis, and wrote and edited the manuscript. Yuanjiang PAN was responsible for funding acquisition, supervision, and validation. Cuirong SUN was responsible for the study design, supervision, funding acquisition, validation, and project administration. All authors have read and approved the final manuscript, and therefore, have full access to all the data in the study and take responsibility for the integrity and security of the data.

Compliance with ethics guidelines

Qirong SHEN, Quan HE, Yuanjiang PAN, and Cuirong SUN declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Supplementary information

Materials and methods; Tables S1-S3; Figs. S1-S10