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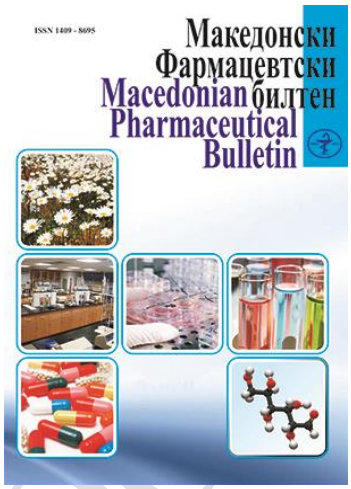


**Title:** A stability-indicating HPLC method for determination of folic acid and its related substances in tablets

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# A stability-indicating HPLC method for determination of folic acid and its related substances in tablets

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## Abstract

In this study, a simple stability-indicating HPLC method for determination of folic acid and its related substances in tablets was optimized and validated. Chromatographic separation was performed on Inertsil C8, 250 x 4.6 mm, 5 μm, using isocratic elution with mobile phase consisting of methanol and phosphate buffer (pH=6,4) (12:88, *v/v* %). The injection volume was 5 μL and the eluted compounds were monitored at 280 nm. The flow rate was 0.7 mL/min and the column temperature was maintained at 30 ˚C. Forced degradation studies were conducted to investigate the stability-indicating ability of the method. The method was validated according to the ICH guideline requirements with respect to selectivity/specificity, linearity, limit of detection, limit of quantification, accuracy, precision and robustness.

The results from the forced degradation study confirmed satisfactory resolution between folic acid and obtained specified impurities (imp. A, imp. C, imp. D, imp. E and PABA) as well as satisfactory resolution between all other formed degradation products, proving that the method is stability-indicating. The optimized and validated method is selective and precise, capable of detecting and quantifying all folic acid related substances. The obtained results from validation parameters imply that the proposed method is suitable

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for its purpose and can be used for routine quantification of folic acid and its related substances in tablets.

**Keywords**: folic acid, related substances, 4-aminobenzoic acid, forced degradation studies

## Introduction

Folic acid (FA), also known as vitamin B9, is a water soluble vitamin used for dietary supplementation as a source of folates, a group of compounds essential for a variety of important physiological functions in humans (Laura Dantola et al., 2018). It is involved in cell multiplication, regulation of gene activity, red and white cell production, renewal of skin and the intestine lining, as well as in the synthesis of chemicals that modulate brain function (Gazzali et al., 2016). The 22$^{nd}$ World Health Organization (WHO) Model List of Essential Medicines lists FA as an antianemic drug in dose strengths ranging from 0.4 mg to 5 mg (Hofsäss et al., 2017; WHO, 2021).

FA is reported as its hydrate in the monograph of the European Pharmacopoeia (Ph. Eur) and British Pharmacopoeia (BP) and as anhydrous substance in US Pharmacopoeia (USP) (BP, 2022; Ph. Eur, 2020; USP, 2020). According to current Ph. Eur monograph, FA has seven specified impurities (imp. A, imp. C, imp. D, imp. E, imp. G, imp. H and imp. I) and two other detectable impurities (imp. B and imp. F). Another impurity which is formed by hydrolytic degradation of FA is 4-aminobenzoic acid (p-aminobenzoic acid, PABA) and is only presented in FA monograph in the British Pharmacopeia (BP, 2022). The chemical structure of FA and its related substances are presented in Fig. 1.

The literature survey revealed several methods for determination of FA content as an individual substance or in combined dosage forms (Brusac et al., 2019; Brusac et al., 2021; Jelicic et al., 2020; Matias et al., 2014). However, only few papers have reported analytical methods for determination of FA in the presence of its related substances in finished pharmaceutical products (Chen et al., 2012; Hongxia et al., 2011; Klaczkow & Anuszewska, 2006; Malenovic et al., 2012). In these studies, all FA impurities were not included and stability-indicating capability of the methods was not presented. Ph. Eur and

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USP report analytical methods for determination of folic acid related substances only for the active substance (Ph. Eur, 2020, USP, 2020). In BP, the described HPLC method for determination of related substances in tablets quantifies only the amount of FA impurity A and the amount of PABA (BP, 2022). According to author's knowledge, no study describes the separation of PABA from the other FA related substances in tablets.

Current regulatory requirements for identification, qualification and control of impurities in drug substances and finished pharmaceutical products require that each impurity is controlled carefully as it may affect the quality, safety and efficiency of finished pharmaceutical products (Bellur Atici et al., 2020; Chatpalliwar et al., 2012). In order to meet the requirements, the content of related substances in finished pharmaceutical products is required to be determined using suitable stability-indicating HPLC methods, as recommended by International Conference on Harmonization (ICH) guidelines (ICH, 2005).

Therefore, the aim of our study was optimization and validation of a simple and reliable stability-indicating HPLC method for simultaneous determination of folic acid and its related substances (imp. A, imp. C, imp. D, imp. E, imp. G, imp. I, imp. H, imp. B, imp. F and PABA) in folic acid tablets.

Fig. 1

## Materials and methods

*Chemicals, reagents and reference substances*

Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, 30% hydrogen peroxide and methanol (HPLC grade) were purchased from Carlo Erba. Sodium carbonate decahydrate was purchased from VWR Chemicals. 85% ortho-phosphoric acid, 37% hydrochloric acid as well as sodium hydroxide pellets were supplied from Fisher Scientific. Demineralized (ultrapure) water, *in-house* prepared with Simplicity system, was used throughout the entire analysis. All the chemicals used were of Ph. Eur grade. Valid batches of reference standards: folic acid CRS, folic acid impurity A CRS, folic acid impurity D CRS, folic acid for system suitability CRS, folic acid for impurity I identification CRS



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were supplied by European Directorate for the quality of Medicines (EDQM). 4-aminobenzoic acid, > 99% was purchased from Sigma Aldrich. Folic acid tablets (5 mg) and placebo were from ReplekFarm, Skopje.

*Equipment*

Chromatographic analyses were performed on a Shimadzu Prominence System and Shimadzu Nexera XR System, both with low pressure gradient (LPG) quaternary pump with degasser, autosampler, controller and PDA detector. Signals were monitored and processed using the Lab solutions software. Photostability studies were carried out in a photostability chamber (BINDER, Germany), whereas thermal stability studies were performed in an air oven (Lilienthal, Germany).

*Chromatographic conditions*

All analyses were carried out using isocratic elution on Inertsil C8 (GL Sciences, Tokyo, Japan), 250 x 4.6 mm, 5 μm, chromatographic column, with a flow rate of 0.7 mL/min. Mobile phase A was methanol, whereas mobile phase B was buffer solution containing 11.16 g/L potassium dihydrogen phosphate and 5.5 g/L dipotassium hydrogen phosphate with pH adjusted to 6.4 with dilute ortho-phosphoric acid (A:B = 12:88, *v/v*, %). The column temperature was maintained at 30 ˚C. The injection volume was 5 μL and analyte peaks were monitored at 280 nm.

*Standard and test solutions*

Stock standard solutions of folic acid (1.0 mg/mL), folic acid for system suitability, SSC solution 1 (contains imp. C, E, G and H, 0.2 mg/mL each), imp. A solution (0.1 mg/mL), imp. D solution (0.04 mg/mL), imp. I solution (0.2 mg/mL), the test solution for assay and related substances and their respective dilutions were prepared according to the Ph. Eur monograph (Ph. Eur, 2020). The stock standard of PABA was prepared according to the BP monograph (BP, 2022). The solution for system suitability control, SSC solution 2, containing imp. C, E, G and H (0.2 mg/mL each), imp. A (0.001 mg/mL), imp. D (0.0004 mg/mL) and PABA (0.001 mg/mL) was prepared in mobile phase. The placebo

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solution was prepared in the same manner as the test solution from excipients, in a quantity that corresponds to their presence in ten mean tablet masses. The test solution and the placebo solution were filtered through 0.45 $\mu$m membrane filters (regenerated cellulose, RC).

*Forced degradation studies*

Forced (stress) degradation studies were performed to test the selectivity of the method i.e., to evaluate the ability of the proposed method to separate FA from its degradation products, according to the procedures given in ICH Guideline Q1A(R2) (ICH, 2003). Intentional degradation was attempted by the stress conditions of photolytic sunlight, with overall illumination of not less than 1.2 million lux/h along with UV energy not less than 200 W h/m$^2$. Powdered tablet mass corresponding to 30 mean tablet masses and placebo samples were kept in an open quartz dish for 13, 25 and 31 days in the chamber. Hydrolytic forced degradation studies were carried out on a water bath, on solutions containing 0.2 mg/mL folic acid and placebo samples under the following conditions: acid (5 M HCl at 60 ˚C for 24 hours), alkaline (1 M NaOH at 60 ˚C for 24 hours) and oxidative conditions (3% $H_2O_2$ at 60 ˚C for 6 hours). Thermal degradation studies were performed in a temperature-controlled oven. The same quantity of powdered tablet mass and placebo powder, as for the photostability testing, were kept at 105 ˚C for 24 hours in the oven.

The peak purity obtained from the stressed samples was verified by using the PDA detector and expressed as peak purity index. The assay of stressed samples was expressed by comparison with reference standards and by calculation of the mass balance (%assay + %sum of all impurities + %sum of all degradation products).

*Method validation*

The method was validated according to the procedures proposed in ICH Guideline Q2A (ICH Q2A, 2005).

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## Results and discussion

*Method validation*

In this study, the stability-indicating HPLC method for determination of folic acid and its related substances in folic acid tablets was validated in respect to selectivity/specificity, linearity, precision and accuracy, as well as robustness of the method and solution stability.

*Selectivity/specificity* - The selectivity represents the ability of the method to differentiate the analyte(s) of interest and other components in the sample matrix. Representative chromatograms of placebo solution, SSC solution 2, imp. I solution and test solution are presented in Fig. 2A, 2B, 2C and 2D, respectively.

Fig. 2

The system suitability testing represents a significant component of any analytical procedure and is used as part of the qualification (Zhang et al., 2020). The chromatogram of the SSC solution 2 (Fig. 2B) revealed that the resolution between folic acid and imp. E was 2.62 and the resolution between imp. A and PABA was 3.90. Also, the obtained value for peak-to-valley ratio between peaks of impurity C and folic acid was 2.43. The obtained value for peak-to-valley ratio between peaks of impurity G and impurity H was 5.25. The obtained results were in accordance with the acceptance criteria of peak-to-valley ratio at least 1.5 and resolution between peaks of FA and imp. E of at least 2.0, resolution between peaks of FA and imp. A of at least 3.0 and resolution between peaks of imp. A and PABA of at least 2.0

Further, the comparison between the representative chromatograms of placebo solution, SSC solution 2, imp. I solution and the test solution revealed that there are no peaks from the placebo that interfere at the retention time of FA, as well as the retention time of the specified impurities.

Forced degradation studies were performed to demonstrate the stability-indicating power and the selectivity of the proposed method. The summary results of the forced-



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degradation study are presented in Table 1 and representative chromatograms in all forced degradation conditions are shown in Fig. 3.

Table 1

Fig. 3

Good separation between the peaks of FA and the peaks of specified impurities, as well as all formed degradation products, in all degradation conditions, were observed. As it can be seen from the results presented in Table 1, the values for the degradation of folic acid under stressed conditions vary between 5 and 20%, which is within the acceptable limits of degradation. Lowest degradation was obtained when folic acid was subjected to photodegradation indicating that the molecule is photo-stable. The peak purity index value was 1.000000, in all degradation conditions. The purity and assay of FA was unaffected by the presence of its impurities and degradation products, thus the method is selective and stability-indicating.

*Linearity and range* - The linearity of the method was determined in the range between 0.01 - 5.91 μg/mL for folic acid, 0.01 - 6.48 μg/mL for impurity A, 0.01 – 1.20 μg/mL for impurity D and 0.05 - 1.50 μg/mL for PABA. The impurity test solutions were prepared at 18 concentration levels for FA, 18 concentration levels for imp. A, 11 concentration levels for imp. D and 10 concentration levels for PABA. Each measurement was performed in triplicate, and the results were evaluated by linear regression analysis using the least squares regression method. The results are shown in Table 2.

Table 2

The results confirmed the linear relationship between peak areas of the examined analytes and the respective concentrations. The correlation coefficient was greater than 0.999 for all the tested impurities. The results indicate very good linearity.

*Limits of detection and quantification* – LOD is defined as the lowest amount of analyte that can be detected but not necessarily quantified as an exact value, whereas limit



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of quantification (LOQ) is defined as the lowest amount of analyte that can be determined with suitable precision and accuracy. Both, LOD and LOQ were determined at a signal-to-noise ration of 3:1 and 10:1, respectively.

*Precision* - Precision of the HPLC stability-indicating method was evaluated through repeatability (system and method repetability) and intermediate precision. The repeatability of the system was evaluated by six determinations of the peak areas of standard solutions containing FA and impurities for which standards are available (imp. A, imp. D and PABA). The obtained RSD values were 0.73, 0.47, 0.15 and 0.73% for FA, imp. A, imp. D and PABA, respectively.

Method repeatability analysis performed on 6 different test solutions showed that RSD for each detected and quantified related supstance was below 1.5%, thus the method is precise. The intermediate precision assessed on two consecutive days, by two different analysts, on two different HPLC systems showed that the RSD value obtained from analysis of 12 test solutions is not more than 10% allowed for intermediate precision, for all the tested impurities, indicating the method is precise (Table 2).

*Accuracy* - Accuracy of the method was evaluated through the study of analytical recovery, at three concentration levels of folic acid, and the specified impurities: imp. A, imp. D and PABA in spiked placebo from tablets. The results are shown in Table 3. The percentages of the obtained recoveries are in the range 98.56 - 102.52%, indicating good accuracy of the method.

Table 3

*Robustness testing*

To determine the robustness of the method, one factor at a time approach was used. Experimental conditions were deliberately changed and resolution between folic acid and imp. E and resolution between PABA and imp. A were recorded. Additionally, peak-to-valley ratio between peaks due to imp. C and folic acid and peaks due to imp. H and imp. G was recorded.



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The flow rate of the mobile phase was evaluated at 0.6 - 0.8 mL/min, the effect of the column temperature was studied at 25 ˚C and 35 ˚C and the effect of the pH of the mobile phase buffer was studied by varying ± 0.1 pH units of buffer's pH (6.4) keeping other mobile phase components constant.

The results from the robustness testing are presented in Table 4. In all the deliberately varied chromatographic conditions the examined impurities were adequately resolved, elution order remained unchanged and the monitored parameters were within the acceptance criteria for system suitability which indicates that the method is robust.

Table 4

*Solution stability*

The solution stability test was performed on standard and test solutions at room temperature. Compared with the initial assay, the difference in the peak areas of the examined related substances at the selected time points should be less than 2%. The % change from determination of peak areas in standard solutions of FA, imp. A, imp. D and PABA after 24 hours of storage at room temperature were 0.44, 0.52, 0.21 and 0.15%, respectively. The % change from determination of peak areas of folic acid in test solution after 24 hours of storage was 0.11% and the % change from determination of peak areas of the examined related substances was below 1% indicating that they were stable up to 24 hours at room temperature.

*Application of the method*

In order to apply the optimized and validated method for determination of folic acid and its related substances, two batches of manufactured tablets were analyzed. The percentage content of PABA was calculated according to BP (BP, 2022), and the percentage content of the other impurities was calculated according to Ph. Eur (Ph. Eur, 2020). The results are presented in Table 5. The results showed that the proposed method is suitable for its intended purpose.



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Table 5

## Conclusion

A simple and precise stability-indicating HPLC method for determination of folic acid and its related substances (imp.A, imp. D, imp. C, imp. E, imp. G, imp. I, imp. H, imp. B, imp. F and PABA) in folic acid tablets was optimized and validated. The validation results showed that the method was accurate, precise, linear, robust and specific. The proposed method can be used for routine analysis of folic acid and its related substances in tablets and also to test the stability of the samples.

## References

Bellur Attici, E., Ağtaş, Ç., Yazal, Y., Ridvanoğlu, N., 2020. Synthesis and characterization of oxitropium bromide related substances and novel stability indication method. J. Pharm. Biomed. Anal. 183, 113145. Available at: https://doi.org/10.1016/j.jpba.2020.113145.

British Pharmacopoeia, 2022. Stationary Office on behalf of the Medicines and Healthcare Products Regulatory Agency (MHRA), 2020. Monograph for folic acid tablets, III-703.

Brusač, E., Jeličič, M., Amidzič Klarič, D., Nigovič, B., Keser, S., Mornar, A., 2021. Development of a HPLC-DAD stability-indicating method and compatibility study of azathioprine and folic acid as a prerequisite for a monolayer fixed-dose combination. Anal. Methods 13, 1433-1431. Available at: https://doi.org/10.1039/D1AY00294E.

Brusač, E., Jeličič, M., Amidzič Klarič, D., Nigovič, B., Turk, N., Klaric, I., Mornar, A., 2019. Pharmacokinetic profiling and simultaneous determination of thiopurine immunosuppressants and folic acid by chromatographic method. Molecules 24(19), 3469. Available at: https://doi.org/10.3390/molecules24193469.

Chatpalliwar, V., Porwal, P., Upmanyu, N., 2012. Validated gradient stability indicating HPLC method for determining diltiazem hydrochloride and related substances in bulk



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drug and novel tablet formulation. J. Pharm. Anal. 2(3), 226-237. Available at: https://doi.org/10.1016/j.jpha.2012.01.003.

Chen, Q., Liu, YL., Luo, YH, Luo, M., Luo, LY., 2012. Determination of folic acid and its related substances of the ferrous fumarate and folic acid dispersible tablet by HPLC. Lat. Am. J. Pharm. 31(4), 555-560.

European Pharmacopoeia. General monographs: folic acid. 10th Ed. 2019-2020. Strasbourg France: European Directorate for the quality of medicines & Healthcare, 2690-2691.

Gazzali M.A., Lobry, M., Colombeau, L., Acherar, S., Azais, H., Mordon, S., Arnoux, P., Baros, F., Vanderesse, R., Frochot, C., 2016. Stability of folic acid under several parameters. Eur. J. Pharm. Sci. 93, 419-430. Available at: https://doi.org/10.1016/j.ejps.2016.08.045.

Hofsäss, M., Souza, J., Silva-Barcellos, N., Bellavinha, C., Abrahamsson, B., Cristofolleti, R., Groot, D.W., Parr, A., Langguth, P., Polli, J., Shah, V., Tajiri, T., Mehta, M., Dressman, J., 2017. Biowaiver monographs for immediate-release solid dosage forms: folic acid. J. Pharm. Sci. 106, 3421-3430. Available at: https://doi.org/10.1016/j.jphs.2017.08.007.

Hongxia, Z., Jinsong, L., 2012. Determination of related substances of folic acid tablets by high performance liquid chromatography. J. Anhiu Agri. Sci. 39(28), 17139-17143.

International Conference on harmonization of technical requirements for registration of pharmaceuticals for human use. ICH Harmonized Tripartite Guideline: Validation of analytical procedures: Text and Methodology Q2(R1). ICH, 2005. Available at: https://www.ema.europa.eu/documents/scientific-guideline/ich-q-2-r1-validation-analytical-procedures-text-methodology-step-5_en.pdf

International Conference on harmonization of technical requirements for registration of pharmaceuticals for human use. ICH Harmonized Tripartite Guideline: Stability testing of new drug substances and drug products Q1A(R2). ICH, 2003. Avilable at: https://www.ema.europa.eu/en/documents/scientific-guideline/ich-q-1-r2-stability-testing-new-drug-substances-products-step-5_en.pdf

Jelicic, M., Brusac, E., Amidzic Klaric, D., Nigovic, B., Turk, N., Mornar A., 2020. A chromatographic approach to development of 5-aminosalicilate/folic acid fixed-dose



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combinations for treatment of Crohns disease and ulcerative colites. Scientific reports 2020, 10, 20838. Available at: https://doi.org/10.1038/s41598-020-77654-2.

Jelicic, M., Brusac, E., Amidzic Klaric, D., Nigovic, B., Keser, S., Mornar, A., 2020. Physicochemical compatibility investigation of mesalazine and folic acid using chromatographic and thermoanalytical techniques. Pharmaceuticals 13(8), 187. Available at: https://doi.org/10.3390/ph13080187.

Klaczkow, G., Anuszevska, E.L., 2006. Determination of impurities in pharmaceutical preparations containing folic acid. Acta Pol. Pharm. 63(5), 391-394.

Laura Dantola, M., Urrutia, M., Thomas, A., 2018. Effect of pterin impurities on the fluorescence and photochemistry of commercial folic acid. J. Photochem. Photobilo B, 181, 157-163. Available at: https://doi.org/10.1016/j.jphotobiol.2018.03.007.

Malenovic, A., Jovanovic, M., Petrovic, S., Kostic N., Vemic, A., Jancic-Stojanovic, B. 2012. Optimization of liquid chromatographic method for the separation of folic acid and its two impurities. Instrum. Sci. Technol. 40, 138-149. Available at: https://doi.org/10.1080/10739149.2011.651670.

Matias, R., Ribeiro, P., Sarraguca, M., Lopes, A., 2014. A UV spectrophotometric method for the determination of folic acid in pharmaceutical tablets and dissolution tests. Anal. Methods 6, 3065-3071. Available at: https://doi.org/10.1039/C3AY41874J.

The United States Pharmacopoeia, the National Formulary (USP43-NP 38), 2020. Rockville, Maryland: United States Pharmacopeial Convention.

World health organization (WHO). WHO model list of essential medicines, 22nd list, 2021. Available at: https://www.who.int/publications/i/item/WHO-MHP-HPS-EML-2021.02. Accessed: May 2, 2022.

Zhang, T., Yin, W., Jin, B., Li, T., Ma, C., 2020. A reliable HPLC-DAD method for simultaneous determination of related substances in TBI-166 active pharmaceutical ingredient. Acta Chrom. 32(2), 80-85. Available at: https://doi.org/10.1556/1326.2019.00517.



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## Резиме

# HPLC метод за следење на стабилноста и определување на фолна киселина и сродни супстанции на фолна киселина во таблети

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**Клучни зборови:** фолна киселина, сродни супстанции, 4-аминобензоева киселина, студии на форсирана деградација

Целта на нашето истражување беше оптимизација и валидација на едноставен HPLC метод, соодветен за следење на стабилноста (stability-indicating), кој овозможува едновремено определување на фолна киселина и сродни супстанции на фолна киселина во таблети. Хроматографското разделување е изведено на хроматографска колона Inertsil C8, 250 x 4.6 mm, 5 μm со изократско елуирање, користејќи мобилна фаза составена од метанол и фосфатен пуфер (pH=6.4) во однос 12:88 (*v/v*, %). Волуменот на инјектирање изнесува 5 μL, а брановата должина на детекција е 280 nm. Протокот на мобилна фаза е 0.7 mL/min, а температурата на колоната 30 ˚C. Способноста на предложениот метод за следење на стабилноста е испитана со примена на студии на форсирана деградација. Валидацијата на методот е изведена во согласност со препораките на ICH водичот за валидација на аналитички



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методи за параметрите селективност/специфичност, линеарност, лимит на детекција, лимит на квантификација, точност, прецизност и робустност.

Во услови на форсирана деградација беше добиена задоволителна вредност за резолуција меѓу фолна киселина и специфицираните онечистувања (онечистување A, C, D, E и 4-аминобензоева киселина), како и за резолуцијата меѓу останатите онечистувања со што методот може да се користи за следење на стабилноста. Оптимизираниот и валидиран метод е селективен и прецизен и со негова примена може да се детектираат и определат сите сродни супстанции на фолна киселина. Резултатите од валидацијата покажаат дека предложениот метод е соодветен за негова намена и може да се користи за рутинско определување на фолна киселина и сродните супстанции на фолна киселина во таблети.



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Table 1. Summary results of the forced-degradation study

| Degradation condition | Time (h) | Assay (% w/w) | Mass balance | % Net degradation | Observation |
|---|---|---|---|---|---|
| Acid degradation (5 M HCl) | 24 | 81.56 | 97.26 | 15.02 | Imp. A, PABA, C, E, D formed |
| Base degradation (1 M NaOH) | 24 | 89.96 | 97.11 | 6.47 | Imp. A, PABA, C, E, D formed |
| Oxidative degradation (3% $H_2O_2$) | 6 | 91.35 | 96.76 | 4.73 | Imp. A, PABA, C, E, D formed |
| Thermal degradation | 24 | 90.47 | 97.56 | 2.01 | Imp. A, PABA, C, E, D formed |
| Photo degradation | 744 | 93.73 | 96.41 | 6.42 | Imp. A, PABA, C, E, D formed |

Mass balance = % assay + % deg. products

% Net degradation = % degradation in stressed sample - % degradation in unstressed sample



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Table 2. LOD, LOQ, Regression and Precision results

| Impurity | LOD (μg mL$^{-1}$) | LOQ (μg mL$^{-1}$) | Regression equation (y) | | Correlation coefficient | Method precision (RSD, %) | Intermediate precision (RSD, %) |
|---|---|---|---|---|---|---|---|
| | | | Slope (b) | Intercept (a) | | | |
| Imp. A | 0.005 | 0.02 | -48.76 | 23557.26 | 0.9999 | 0.57 | 2.47 |
| Imp. D | 0.004 | 0.01 | 62.19 | 46124.37 | 0.9999 | 0.66 | 2.48 |
| PABA | 0.016 | 0.05 | -59.93 | 31930.69 | 0.9992 | ND* | ND* |
| Imp. C | NA | NA | NA | NA | NA | 0.80 | 2.34 |
| Imp. E | NA | NA | NA | NA | NA | 0.87 | 4.61 |

*PABA was not detected in test solutions prepared for method/intermediate precision evaluation

*NA – not applicable



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Table 3. Results from the testing of the accuracy of the method

| Amount spiked | % Recovery* | | | |
|---|---|---|---|---|
| | Folic acid | Imp. A | Imp. D | PABA |
| 50% | 99.57 ± 0.26 | 100.79 ± 0.06 | 102.52 ± 0.03 | 100.76 ± 0.06 |
| 100% | 99.47 ± 0.05 | 99.56 ± 0.01 | 101.65 ± 0.01 | 100.50 ± 0.01 |
| 150% | 98.56 ± 0.04 | 100.17 ± 0.01 | 100.99 ± 0.01 | 101.65 ± 0.01 |

*Results are expressed as mean ± RSD (%) for three determinations at each level



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Table 4. Results from robustness testing

| Criteria | | Resolution between FA and imp. E | Resolution between imp. A and PABA | Peak to valley ratio: Imp. C and FA | Peak to valley ratio: Imp. G and Imp H |
|---|---|---|---|---|---|
| Change in flow rate (mL/min) | 0.6 | 2.58 | 3.01 | 1.73 | 4.46 |
| | 0.7 | 2.62 | 3.90 | 2.43 | 5.25 |
| | 0.8 | 2.18 | 2.95 | 1.59 | 4.76 |
| Change in column temperature (˚C) | 25 | 2.29 | 2.89 | 1.70 | 4.88 |
| | 30 | 2.62 | 3.90 | 2.43 | 5.25 |
| | 35 | 2.05 | 2.17 | 1.53 | 4.23 |
| Change in buffer pH value | 6.30 | 2.43 | 2.60 | 1.93 | 3.96 |
| | 6.40 | 2.62 | 3.90 | 2.43 | 5.25 |
| | 6.50 | 2.34 | 2.58 | 1.74 | 7.90 |



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Table 5. Results from determination of folic acid and related substances in production batches of folic acid tablets

| Impurity | Production batch 1 | Production batch 2 |
|---|---|---|
| FA (%) | 100.41 | 99.74 |
| Imp. A (%) | 0.20 | 0.15 |
| PABA (%) | ND* | ND* |
| Imp. D (%) | 0.13 | 0.21 |
| Imp. C (%) | 0.10 | 0.08 |
| Imp. E (%) | 0.08 | 0.15 |
| Imp. G (%) | ND | ND |
| Imp. H (%) | ND | ND |
| Imp. I (%) | ND | ND |
| Unspecified impurities (%) | 0.04; 0.04 | 0.02, 0.04 |
| Total (%) | 0.59 | 0.65 |

*ND – not detected



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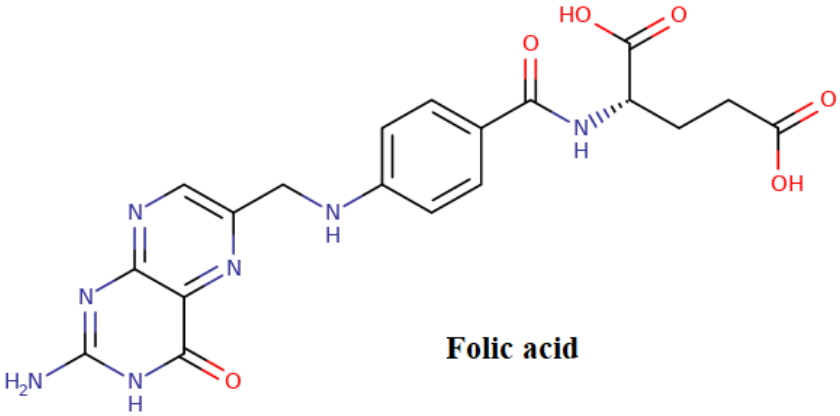
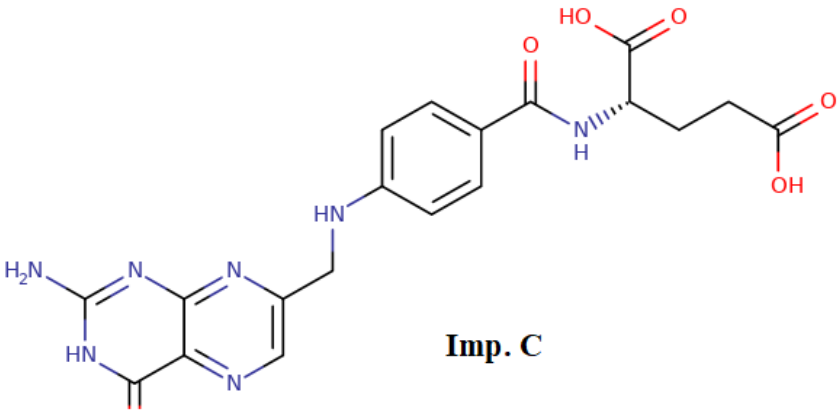
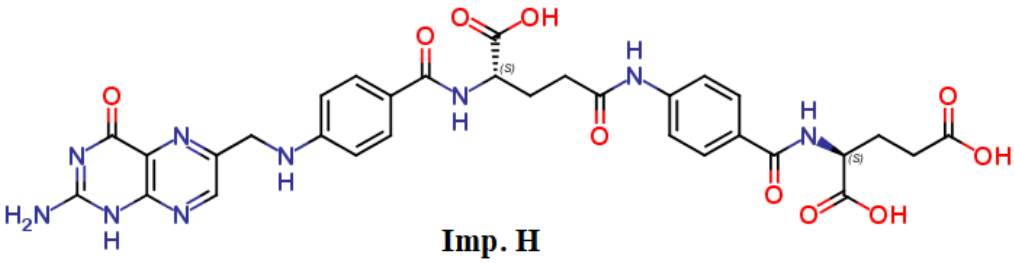


**Folic acid**



**Imp. C**



**Imp. H**

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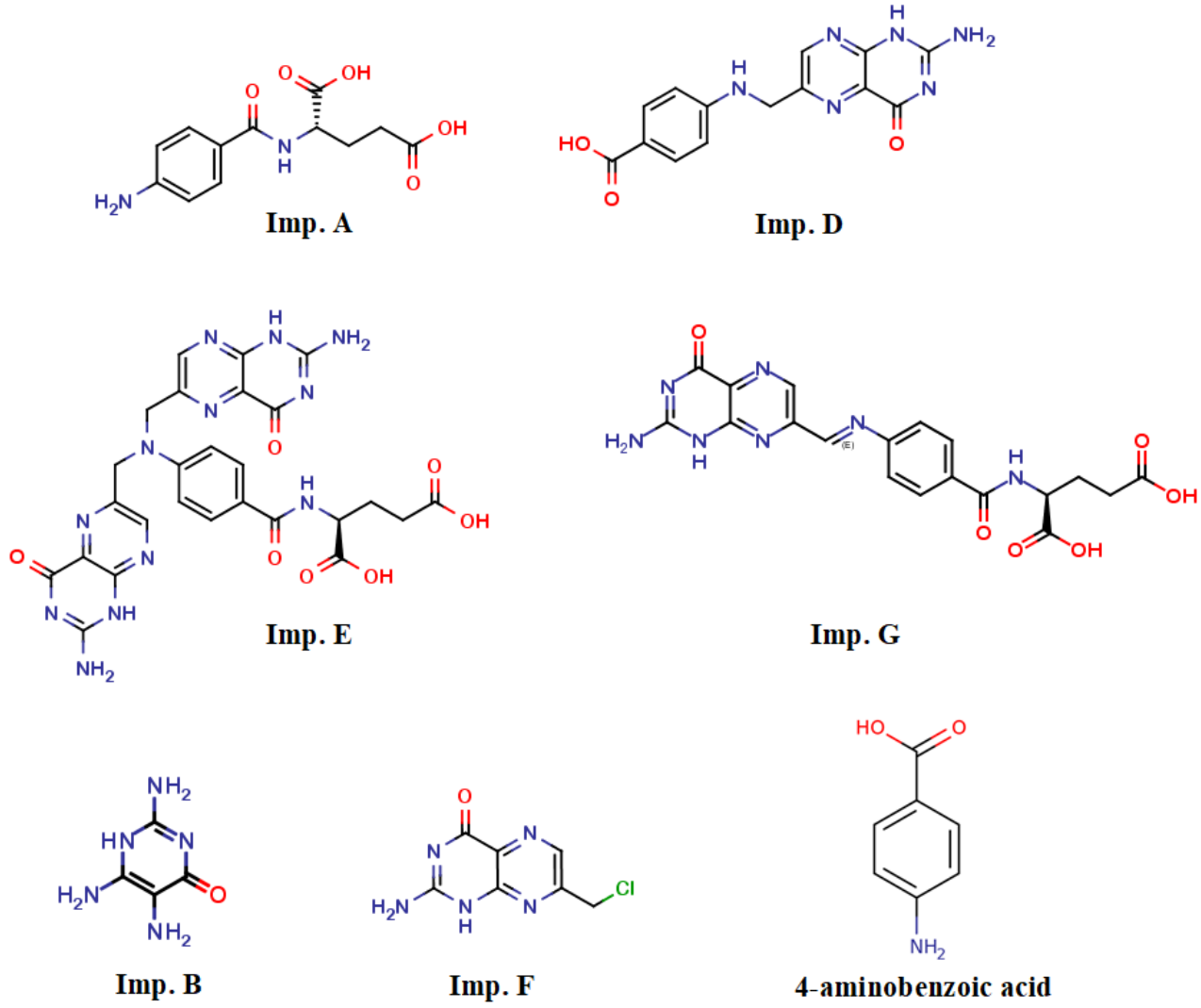


Fig. 1. Chemical structures of FA and its related substances.

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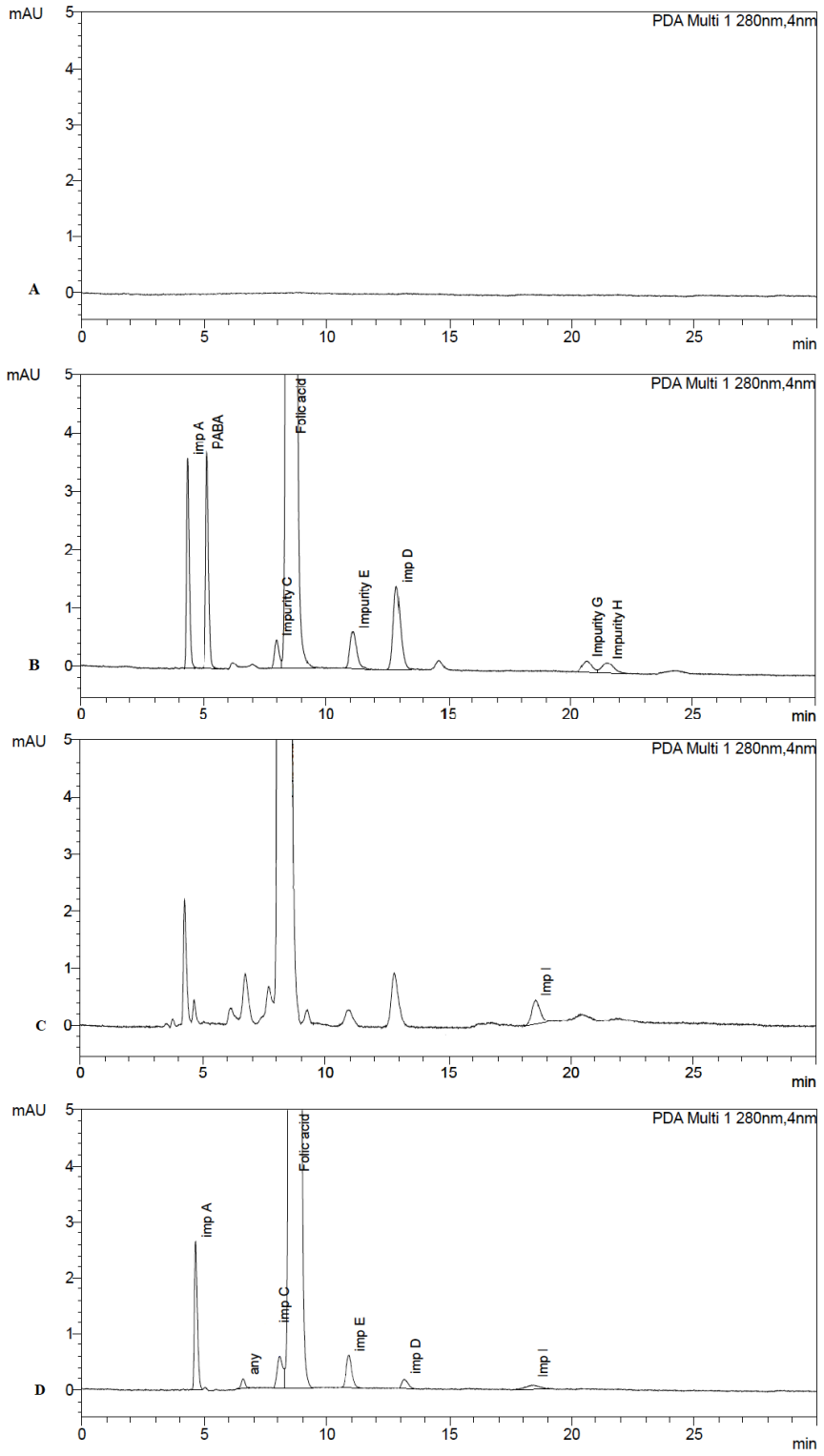


Fig. 2. Representative chromatograms of: A) Placebo solution, B) SSC solution 2, C) Impurity I solution, D) Test solution.

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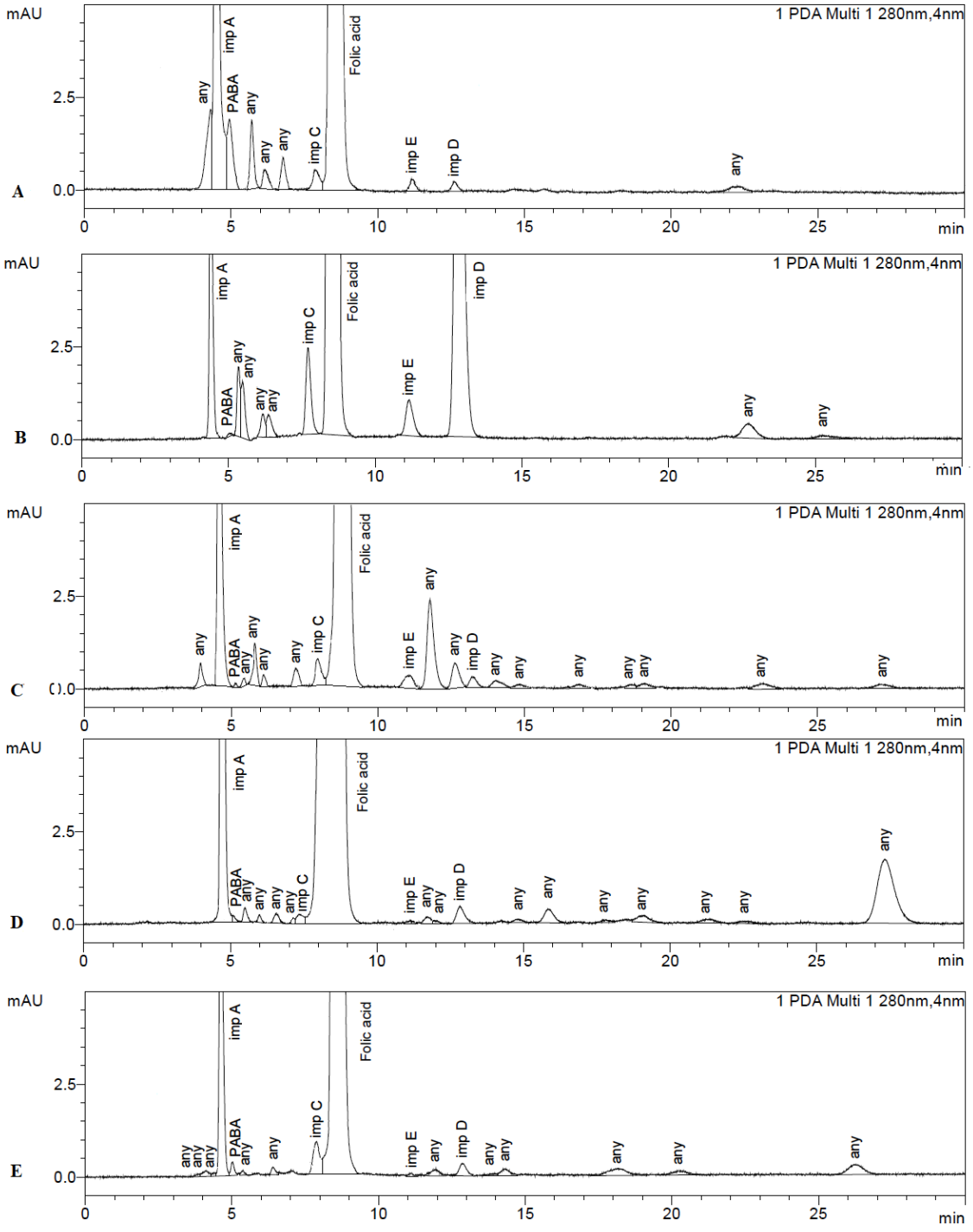


Fig. 3. Representative chromatograms of forced degradation study conditions A) Base degradation B) Acid degradation C) Oxidative degradation D) Thermal degradation E) Photodegradation.

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