Folic (pteroglutamic) acid belongs to the group of B vitamins. It is metabolized in the body to its active form, tetrahydrofolic acid, which is involved in many biochemical reactions. Among other reactions, it participates in the synthesis of purine and pyrimidine bases, DNA synthesis and amino acid metabolism, especially that of homocysteine, the deficit of which contributes to the development of ischaemic heart disease. It plays an important role in the tissues in which cell divisions take place, especially in the hematopoietic system and the gastrointestinal epithelium (1-3). Folic acid has an increasing significance in the prevention of neural tube defects occurring in the third and fourth week of development of the human embryo (4, 5). It is an active substance of many pharmaceutical products, vitamin and vitamin-mineral preparations, especially those used by women before conception and in the early phase of pregnancy.

Folic acid is usually obtained in a condensation reaction of an appropriate pyrimidine with an alkaline three-carbon molecule and N-4-aminobenzoyl-L-glutamic acid (a single step process). It can also be obtained as a result of two step synthesis consisting in the production of the pterin part first, and then condensation with N-4-aminobenzoyl-L-glutamic acid. Another variant of two step synthesis (more complex) is the condensation of 2,4,5-triamino-6-hydroxypyrimidine with N-[p-(2,3-dihydroxy-2-en-propylidene)-benzoyl]-glutamate or N-acetyl-N-(2,3-dibromopropyl)-4-aminobenzoylglutamic acid.

The possible impurities of folic acid are: p-aminobenzoic acid and N-4-aminobenzoyl-L-glutamic acid (products of hydrolysis).

The purpose of our study was to develop a simple, fast and sensitive analytical method to separate these impurities, identify them and determine their quantity in the selected single-component pharmaceutical products for which such tests have not been performed (only the folic acid content was determined using either the microbiological method or the high-performance liquid chromatography method).

When determining the folic acid content in pharmaceutical products using the HPLC method, the test of impurities from the active substance cannot be performed simultaneously, as the extraction methods of folic acid and the impurities tested are different. The chromatographic conditions of the compounds analyzed also differ (wavelengths at which the determinations are performed and mobile phases). Neither Ph.Eur., BP nor USP specify any methods for testing impurities in folic acid tablets. According to USP, only the folic acid content in tablets is tested (HPLC method), and Ph.Eur. and BP describe the purity testing in detail but only for the active substance.

The folic acid impurities testing (using the HPLC method) is described in the Polish Pharmacopeia VI (FP VI). According to its requirements, the limit of impurities for p-aminobenzoic acid has been determined at the level of 0.5%, and for N-4-aminobenzoyl-L-glutamic acid at the level of 2%.

The development of a new analytical method was advisable because the proposed method could represent an alternative for the method described in FP VI. Its use could contribute to improved quality of pharmaceutical products.

**EXPERIMENTAL**

**Equipment**

A Shimadzu liquid chromatograph with LC-10 AT pump, SCL – 10A VP control system, SIL – 10 AD VP autosampler, SPD – 10 AV VP spectrophotometric detector, DGU – 14 A degasser and a computer with CLASS – VP software (version 5.3).

**Standards**

Folic acid – purity 97.7%, water content 8%; p-aminobenzoic acid – purity 99.8%; N-4-aminobenzoyl-L-glutamic acid – purity 99.5% all from Sigma.

**Reagents**

Potassium dihydrophosphate, (Sigma), water for HPLC, methanol for HPLC (Lab-Scan), tetrabutylammonium hydroxide 40% water solution (Aldrich), 85% phosphoric acid (BDH), ammonia water 25% (P.P.H. “Standard”).

**Pharmaceuticals**

Folovit tablets 0.4 mg, Polfarmex, Poland, Acidum folicum 5 mg, Hasco-Lek, Poland.
Chromatographic analysis conditions

Spectrophotometric detector, λ = 269 nm, column WATERS SPHERISORB SSODS1, 250 x 4.6 mm, column temperature: 30°C, volume injected onto the column – 25 µL, mobile phase: 2 g of KH₂PO₄ was dissolved in 640 mL of water, 15 mL of tetrabutylammonium hydroxide 0.5 mol/L methanol solution and 270 mL of methanol was added. The pH value of the mixture was adjusted to 5.0 using 1 mol/L phosphoric acid and filled up with water to the volume of 1 litre, mobile phase flow – 1.2 mL/min

Chromatographic system testing

Resolution

To check the resolution of p-aminobenzoic acid and N-4-aminobenzoyl-L-glutamic acid peaks, a solution consisting of a mixture of the following two standards was prepared: 0.5 µg/mL p-aminobenzoic acid and 2 µg/mL N-4-aminobenzoyl-L-glutamic acid in the mobile phase. The solution was injected onto the column six times (Figure 1).

Measurement of repeatability

To determine the precision of the HPLC system used, two standard solutions were prepared: a) p-aminobenzoic acid, 0.5 µg/mL, b) N-4-aminobenzoyl-L-glutamic acid, 2 µg/mL. Each of these solutions was injected onto the column six times. Chromatograms were recorded with peak area values for each standard. The results are presented in Table 1.

<table>
<thead>
<tr>
<th>Pharmaceutical product</th>
<th>p-aminobenzoic acid content [%]</th>
<th>N-4-aminobenzoyl-L-glutamic acid content [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folovit 0.4 mg</td>
<td>0.101</td>
<td>0.298</td>
</tr>
<tr>
<td></td>
<td>0.103</td>
<td>0.295</td>
</tr>
<tr>
<td></td>
<td>0.100</td>
<td>0.292</td>
</tr>
<tr>
<td></td>
<td>0.102</td>
<td>0.297</td>
</tr>
<tr>
<td></td>
<td>0.101</td>
<td>0.289</td>
</tr>
<tr>
<td></td>
<td>0.100</td>
<td>0.294</td>
</tr>
<tr>
<td>Acidum folicum 5 mg</td>
<td>0.106</td>
<td>0.119</td>
</tr>
<tr>
<td></td>
<td>0.109</td>
<td>0.120</td>
</tr>
<tr>
<td></td>
<td>0.108</td>
<td>0.118</td>
</tr>
<tr>
<td></td>
<td>0.108</td>
<td>0.121</td>
</tr>
<tr>
<td></td>
<td>0.107</td>
<td>0.120</td>
</tr>
<tr>
<td></td>
<td>0.106</td>
<td>0.117</td>
</tr>
</tbody>
</table>

Figure 1. Chromatogram of the mixture of standards.

peak nr 1 – p-aminobenzoic acid (conc. 0.5 µg/mL)
peak nr 2 – N-4-aminobenzoyl-L-glutamic acid (conc. 2 µg/mL)
p-Aminobenzoic acid standard calibration curve

To determine whether the proposed conditions are optimal and can be used for the tests, the relationship between detector indications and the amount of p-aminobenzoic acid placed onto the column was evaluated. The following p-aminobenzoic acid solutions in the mobile phase were prepared: 0.2 µg/mL, 0.3 µg/mL, 0.4 µg/mL, 0.6 µg/mL, 0.8 µg/mL and 1.0 µg/mL. Each of these solutions was injected onto the column.

N-4-aminobenzoyl-L-glutamic acid standard calibration curve

For N-4-aminobenzoyl-L-glutamic acid, six standard solutions in the mobile phase of the following concentrations were prepared: 0.8 µg/mL, 1.2 µg/mL, 1.6 µg/mL, 2.0 µg/mL, 2.4 µg/mL and 3.2 µg/mL. Each of these solutions was injected onto the column.

Determination of p-aminobenzoic acid and N-4-aminobenzoyl-L-glutamic acid content in the selected pharmaceutical products

Sample preparation for the tests

Equal amounts of tablet mass, corresponding to 2.5 mg of folic acid, were weighed and placed into 25 mL calibrated flasks. Next, 0.25 mL of 12% ammonium hydroxide and 15 mL of the mobile phase were added. The mixture was sonicated for 10 minutes and shaken for 15 minutes. The flasks were made up to the mark with the mobile phase and filtered. Six independent samples were prepared for each product. Each sample was injected onto the column three times (Figure 2 and 3). The percent content of impurities in the analyzed products is presented in Table 1.
Preparation of the standard solutions of the impurities

Standard solutions of p-aminobenzoic acid and N-4-aminobenzoyl-L-glutamic acid.

Two standard solutions in the mobile phase were prepared: – 5 µg/mL p-aminobenzoic acid (solution A), – 10 µg/mL N-4-aminobenzoyl-L-glutamic acid (solution B).

Mixture of standards

1 mL of solution A and 2 ml of solution B were pipetted into a 10 ml calibrated flask. The final concentrations of p-aminobenzoic acid and N-4-aminobenzoyl-L-glutamic acid were 0.5 µg/mL, and 2 µg/mL, respectively.

Preparation of the standard solution of folic acid

10 mg of folic acid was weighed and placed into a 10 mL calibrated flask. 0.1 mL of 12% ammonium hydroxide and 6 mL of the mobile phase were added. The mixture was sonicated for 5-10 minutes and shaken for 10-15 minutes. From this solution, 1 mL was pipetted into a 10 mL calibrated flask and made up to the mark with the mobile phase.

The final concentration of folic acid was 0.1 mg/mL.

The folic acid solution was prepared for active substance identification on the chromatogram of the sample (retention time of folic acid tR ~10 min).

RESULTS AND DISCUSSION

The proposed method for the identification and determination of impurities allows for satisfactory separation of p-aminobenzoic acid and N-4-aminobenzoyl-L-glutamic acid. The separation coefficient was Rs = 4.45. Peak asymmetries (10%) for p-aminobenzoic acid and N-4-aminobenzoyl-L-glutamic acid were 1.32 and 1.28, respectively. The number of theoretic plates was N = 5092.

On the basis of the statistic evaluation performed it was found that the repeatability of measurements was satisfactory. The relative standard deviation (RSD) for p-aminobenzoic acid (retention time tR ~ 4.1 min) was 0.96%, and for N-4-aminobezoyl-L-glutamic acid (retention time tR ~ 5.3 min) – 0.68%.

The relationship between detector indications and acid concentrations is linear: for p-aminobenzoic acid, in the concentrations range of 0.2 µg/mL to 1.0 µg/mL (R² = 0.9995), and for N-4-aminobenzoyl-L-glutamic acid in the range of 0.8 µg/mL to 3.2 µg/mL (R² = 0.9999).

For p-aminobenzoic acid, the limit of detection (LOD) was 23 ng/mL, and the limit of quantitation (LOQ) was 81 ng/mL. For N-4-aminobenzoyl-L-glutamic acid, these values were: LOD = 20 ng/mL and LOQ = 70 ng/mL.

In addition to the peaks of the impurities, also other peaks were seen on the chromatograms of the samples (Figure 2 and 3). As placebo could not be obtained from the manufacturers, other peaks could not be identified.

The repeatability of the described method is good as evidenced by the relative standard deviation values for both p-aminobenzoic acid and N-4-aminobenzoyl-L-glutamic acid (Table 1).

Statistical analysis showed that the precision of the proposed method is satisfactory, as evidenced by low relative standard deviation values which is the measure of the analytical statistic error.

In view of the simple method of sample preparation for the tests and very short retention times of the determined impurities, this method can be used in pharmaceutical analysis and can represent an alternative to the method presented in FP VI.

The introduction of the described method in the tests of pharmaceutical products for which such tests have not been performed could improve the quality of these products in the future. In fact, the presence of impurities above the allowable limits can have a significant effect on the development of human foetus, as well as the health of patients who take pharmaceutical products containing folic acid.

REFERENCES