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A validated ultra high pressure liquid chromatographic method for qualification and quantification of folic acid in pharmaceutical preparations

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ABSTRACT

A fully validated UHPLC method for the identification and quantification of folic acid in pharmaceutical preparations was developed. The starting conditions for the development were calculated starting from the HPLC conditions of a validated method. These start conditions were tested on four different UHPLC columns: Grace Vision HT™ C18-P, C18, C18-HL and C18-B (2 mm × 100 mm, 1.5 μm). After selection of the stationary phase, the method was further optimised by testing two aqueous and two organic phases and by adapting to a gradient method. The obtained method was fully validated based on its measurement uncertainty (accuracy profile) and robustness tests.

A UHPLC method was obtained for the identification and quantification of folic acid in pharmaceutical preparations, which will cut analysis times and solvent consumption.

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1. Introduction

Folic acid, also called vitamin M, B9 or B11, is a water soluble vitamin, which is essential for humans. Folic acid is present, as folate, in several vegetables and citrus fruits, but is very sensitive to light and oxygen. This is one of the reasons why there is a risk of deficiency in the western population. Even if it is proven that when a supplementation of mixed fruits and vegetables is administered, the serum concentration of folate is increased [1], the majority of the western population does not attain the recommended daily intake (RDI) of 200 μg through their diet. This is the reason why folic acid is present in the majority of the nutritional vitamin supplements sold across the world. The European Union permits the enrichment of for example bread and cereals with folic acid and in the United States, such an enrichment is even obliged for cereals with a minimum level of 25% of the recommended daily intake (RDI) [2].

Folic acid has different important functions in the human body. It plays a major role in the synthesis of red blood cells, in the formation of RNA and DNA, in the development of tissues and the brain of the foetus and the growth of a baby [3]. Folic acid is also possibly a protective factor for several pediatric tumors [4]. It is also considered that it has a role in the prevention of cardiovascular diseases, since a higher serum folate concentration decreases the concentration of homocystein [5].

Due to the importance of folic acid, their concentration in nutrition supplements, pharmaceutical preparations, infant formulations and food [6,7] is regularly checked by the competent authorities.

The United States Pharmacopoeia describes a HPLC method for the quantification of folic acid in tablets [8], but does not take into account that folic acid is often present in formulations containing other water soluble vitamins and nutritional supplements.

In literature several analytical methods can be found to quantify folic acid. The most important of them use liquid chromatography. Different methods were published using different detection systems like UV [9–12], fluorescence detection [10] and mass spectrometry [6,7,13,14].

In this paper an ultra high pressure liquid chromatography (UHPLC) method was developed for the qualification and the quantification of folic acid in pharmaceutical preparations. One of the main advantages of UHPLC is that it is able to cut analysis times and solvent consumption compared to HPLC.

In a first step of the development the starting conditions for UHPLC were calculated based on a validated HPLC method, used routinely in our laboratory for the quantification of folic acid. The starting conditions were tested on four different columns and the column giving the best resolution and peak symmetry was used for further optimisation of the method.

The obtained method was eventually validated for its performance as routine method for the qualification and quantification

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of folic acid in pharmaceutical preparations. The method was also compared with the original HPLC reference method.

2. Methods and materials

2.1. Standards

The reference standard for ascorbic acid (vitamin C) (batch 08L19-B03-240178) was purchased from Fagron (Waregem, Belgium). For nicotinamide (vitamin B_3) (batches 1143744 and 24305088), folic acid (vitamin B_9) (batches 1335713 and 21808145) and thiamine hydrochloride (vitamin B_1) (batches 1166292 and 33905167) the standards were purchased from Fluka (Steinheim, Germany) and for riboflavine (vitamin B_2) (batches 47861 and LB68157) and pyridoxine hydrochloride (vitamin B_6) (batches 47862 and LB58889) from Supelco (Bellefonte, Pennsylvania, USA).

2.2. Reagents

Acetonitril and methanol were purchased from Biosolve (Valkenswaard, The Netherlands) and formic acid was purchased from VWR International (Leuven, Belgium). Ammonia solution 28% was purchased from Vel (Leuven, Belgium) and ammonium thiocyanate and glacial acetic acid were purchased from Merck (Darmstadt, Germany). Sodium hexane sulfonate monohydrate was purchased from Across Organics (Geel, Belgium).

2.3. Equipment

Method development and validation was performed on an Acquity UPLC™ system (Waters, Milford, USA). The system consisted of a binary solvent manager, a sample manager and a photo diode array detector. The output signal was monitored and processed using the Empower2 software.

The HPLC reference method was performed on an Alliance 2690 HPLC system (Waters, Milford, USA) coupled to a 966 PDA detector (Waters, Milford, USA). The output signal was also monitored and processed with the Empower2 software.

2.4. Chromatographic conditions

Initial screenings were performed on four C18 columns: Grace Vision HT™ C18-P, C18, C18-HL and C18-B, all with dimensions 2 mm × 100 mm, 1.5 μm (Grace Davision Discovery Sciences, Lokeren, Belgium). The four columns have a different affinity for hydrophobic and polar components due to differences in endcapping. Method optimisation and validation was performed on the Grace Vision HT™ C18-column.

During the first screenings isocratic settings were used based on the parameters calculated by the Acquity UPLC™ columns calculator software (Milford, MA, USA). Only during optimisation it was decided to switch to gradient elution.

During optimisation mQ-gradient water (produced by a mQ-gradient A10, Millipore, Billerica, USA) and a 0.1% formic acid solution were used as aqueous phase and acetonitril or methanol as organic phase. As strong needle wash solvent a mixture of 80% organic modifier and 20% mQ-gradient water was used and as weak needle wash solvent 80% mQ-gradient water and 20% organic modifier (acetonitril or methanol depending on the screening experiment applied).

The HPLC analysis was performed on a platinum C18 column (150 mm × 3 mm, 5 μm) (Alltech-Grace, Deerfield, USA). The mobile phase consists of a solvent containing 50 ml of a solution of 4.3 g sodium hexane sulfonate in 100 ml acetonitril/water 50/50, adjusted to pH 2.65 with glacial acetic acid in 1000 ml of a mixture of 23.4/76.6 methanol/water. The analysis was performed in isocratic mode.

2.5. Sample preparation

2.5.1. Preparation of standards

Calibration standards were prepared by bringing 30 mg of folic acid in 5 ml of a working solution, containing 7 ml of a 28% ammonia solution and 7.6 g ammonium thiocyanate for 100 ml water, to ensure the stability of folic acid in solution. The solution was brought to a volume of 50 ml with a mixture of 25/75 methanol/water. Starting from this solution, standards were prepared with concentrations 0.6 μg/ml, 1.2 μg/ml, 3.6 μg/ml, 6.0 μg/ml and 12.0 μg/ml.

Following the same protocol a control standard was prepared with a concentration of 4.8 μg/ml.

2.5.2. Preparation of samples

For the preparations of the samples a matrix was prepared containing 17 mg vitamin B1, 19 mg vitamin B2, 217 mg vitamin B3, 24 mg vitamin B6 and 723 mg ascorbic acid for 1 g of powder.

A stock solution of 60.4 μg/ml folic acid was prepared following the protocol of the standards.

For the samples 250 mg of the matrix was brought in a volumetric flask of 100 ml and 5 ml working solution was added. Stock solution was added in such a way that samples were obtained with concentration of 1.16 μg/ml, 1.75 μg/ml, 3.49 μg/ml, 4.66 μg/ml and 6.98 μg/ml. The solutions were brought to volume with 25/75 methanol/water and centrifuged.

2.6. Experimental design

The robustness testing of the method was performed using experimental design. A three-factor three-level full factorial design was performed [15]. The experiments were performed three times and the interpretation of the effects of the different factors was performed using regression. A quadratic response surface area was constructed, represented by following general equation:

$$y = b_0 + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x_2 \quad (1)$$

where b_0 represents the intercept, b_i and b_{ij} represent the regression coefficients and x_i represents the factors tested. The significance of the regression coefficients is a value for the significance of the effects of the different factors on the response. The regression coefficients of the products of two factors represent the significance of the interaction effects of the two factors [15].

2.7. Method validation

The method validation was performed in accordance with the ISO17025 guideline using the total error approach [16–19].

The developed method was compared to the HPLC-method which was considered as the reference method. This comparison was done following the statistical protocol described by Kuttatharmmakul et al. [20].

2.8. Statistics

The statistical analysis was performed using Statgraphics Plus 5.1 (STSC Inc., Rockville, MD, USA) and Microsoft Excell 2003.

3. Results

3.1. Selection of the system to be optimised

In a first step of the method development the HPLC-conditions of a validated method were recalculated to UHPLC conditions with the software Acquity UPLC™ columns calculator (Waters, Milford, USA). This program estimates the UHPLC parameters, based on the efficiency calculated from the introduced HPLC parameters.

Parameters of the HPLC-method entered into the program were column dimensions 150 mm × 3.0 mm, molecular weight for folic acid 441 g/mol, injection volume 7 μl, column temperature 27 °C and flow 0.7 ml/min.

Since the HPLC conditions used were for an isocratic method, the resulting UHPLC parameters were also for an isocratic separation.

Since it was decided to simplify the mobile phase for UHPLC these calculations were only used as a basis to obtain start conditions for the development. The mobile phase was changed since the small amounts of sodium hexane sulfonate and acetonitril in the HPLC mobile phase are present to improve peak symmetry of the compounds on the used HPLC column. Since the same column for UHPLC did not exist it was necessary to develop a completely new method and so there was no reason to add these components to the mobile phase. Based on the calculated values following start conditions were chosen: column 100 mm × 2.1 mm, 1.7 μm particles, flow 0.4 ml/min, column temperature 40 °C, injection volume 1.1 μl and a mobile phase consisting of 80% milli Q-gradient water and 20% of methanol. This mobile phase was chosen since it is more adapted to be used in UHPLC.

These conditions were tested on four UHPLC-columns: Vision HT C18, C18-P, C18-B and C18-HL. These columns were selected since the HPLC-method was developed on a platinum C18 column (150 mm × 3.0 mm, 5 μm) and an analogue column is unavailable for UHPLC. The columns were tested for the separations of six vitamins: ascorbic acid, folic acid, Vitamin B1, B2, B3 and B6. The chromatograms were also evaluated for the peak shape of folic acid.

Columns C18 and C18-B gave the best separations, although not all vitamins were separated.

In a next step a three-factor two-level full factorial design was performed. The factors were the stationary phase (C18 or C 18-B), the aqueous phase (milli Q-gradient water or 0.1% formic acid) and the organic phase (acetonitril or methanol). The same chromatographic parameters as mentioned above were applied. Visual inspections of the chromatograms lead to the conclusion that only the combination of the C18-column with a mobile phase consisting of 90/10 0.1% of formic acid/acetonitril with a flow of 0.4 ml/min and a column temperature of 40 °C gave a reasonable separation of folic acid, and therefore it considered the optimal isocratic UHPLC method. This method was used for further optimisation with a gradient method. Fig. 1 shows the corresponding chromatogram.

3.2. Optimisation of the method

Fig. 1 shows that even though folic acid is separated from the other vitamins, vitamin C and B1 and vitamin B6 and B3 are not separated. Also the symmetry of the folic acid peak should be improved.

Since the not separated vitamins have low retention times it was decided to decrease the flow from 0.4 ml/min to 0.3 ml/min and to increase the percentage of aqueous phase in the beginning of the gradient from 90% (isocratic condition) to 98% and to keep this composition for a few minutes in order to separate the first eluting peaks. In a next step a gradient was started in order to elute folic acid and vitamin B2.

Table 1 shows the final gradient method and Fig. 2 shows the corresponding chromatogram.

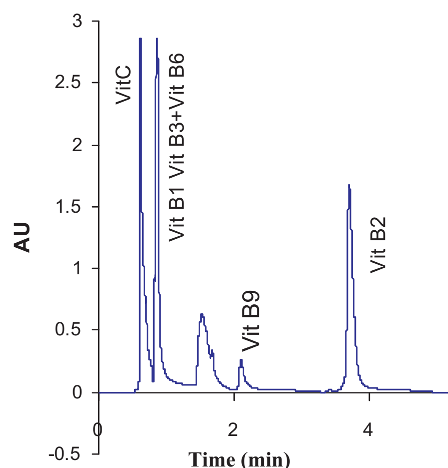

Fig. 1. Chromatogram obtained under isocratic conditions for the separations of folic acid and other water soluble vitamins.

From Fig. 2a it can be seen that all vitamins are now separated. The symmetry factor for folic acid was 0.89. As example Fig. 2b shows a chromatogram obtained for a commercial sample. The sample consisted of tablets containing 100 μg of folic acid in a real sample matrix. This method was validated following the ISO 17025 requirements in order to implement it in the routine analysis of folic acid in pharmaceutical preparations.

3.3. Validation

3.3.1. Selectivity

The selectivity of detection was ensured by determining the retention time of each vitamin separately and by monitoring the UV-spectra of the different vitamins during the different analyses.

3.3.2. Linearity of the calibration line

Five calibration standards for folic acid were prepared in order to evaluate the relationship between the area under the curve and the concentration. The linearity of the relationship was evaluated in a concentration range of 0.6–12 μg/ml, covering the normal range of concentrations obtained when analyzing pharmaceutical preparations.

The calibration curve was obtained using least-square linear regression and the linearity was confirmed with a R^2 value of 0.9999 and a quality coefficient of 0.817% [21].

3.3.3. Trueness, precision, accuracy and uncertainty assessment

A statistical approach based on the total error profiles was applied to validate the method.

Spiked blank samples at five concentration levels were used. Every sample was prepared in triple and analysed for five consecutive days. The preparation and the exact concentrations of the samples are described in Section 2.5.2.

The concentrations of the spiked samples were back-calculated using a calibration line, prepared as described in Section 2.5.1, to determine the linearity between theoretical and measured concen-

Table 1
The optimised gradient for the UHPLC method.

Time	Flow	0.1% formic acid	Acetonitril
0 min	0.3 ml/min	98%	2%
2 min	0.3 ml/min	98%	2%
3 min	0.3 ml/min	80%	20%
4 min	0.3 ml/min	80%	20%
5 min	0.3 ml/min	98%	2%

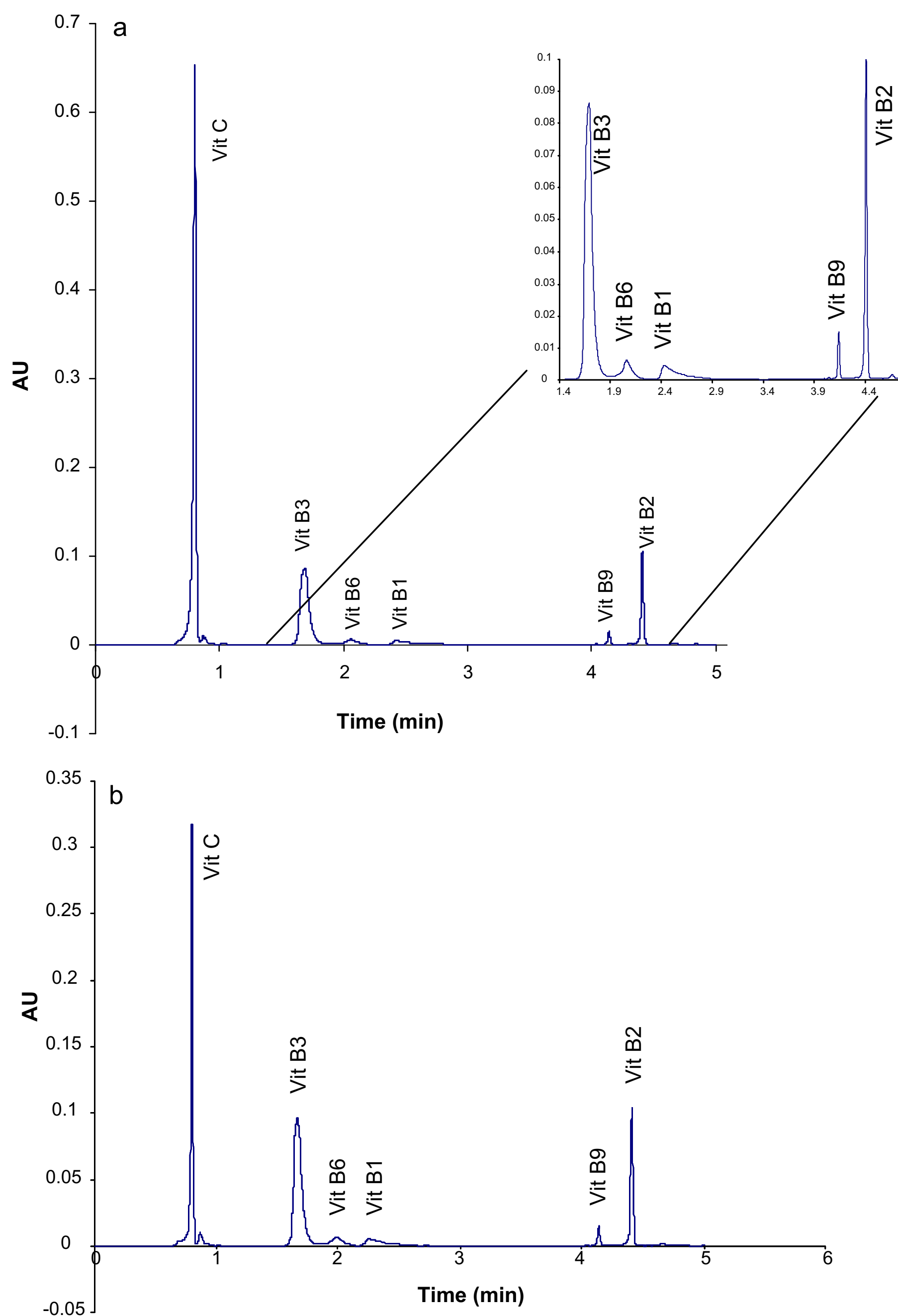

Fig. 2. (a) Chromatogram obtained under gradient conditions for the separations of folic acid and other water soluble vitamins. (b) Example of a chromatogram obtained for a real commercial sample/matrix.

trations, the mean relative bias, the repeatability, the intermediate precision and the β-expectation tolerance limits at the 5% level. All results are shown in Table 2.

The relationship between the theoretical and the measured concentrations is clearly linear with an R^2-value near to 1.

Trueness refers to the closeness of agreement between the obtained values and the known exact concentration of the spiked samples and is a measure for the systematic errors of the method [22]. It is expressed in terms of relative bias. From Table 2 it can be concluded that the trueness for folic acid is acceptable since the relative bias is always smaller than 1%.

The precision is a measure for the relative errors of the method and is expressed as the relative standard deviations (RSD) for repeatability and intermediate precision. From Table 2 it can be seen that a good precision is obtained since the maximal RSD obtained is of 2.06%.

Accuracy takes into account the total error of the test results and is represented by the β-expectation tolerance intervals. The acceptance limits were set at 10%. As shown in Table 2 and Fig. 3 the relative β-expectation tolerance intervals did not exceed the acceptance limits, which means that each future measurement of unknown samples will be included in the tolerance limits at the 5% level.

The uncertainty represents the dispersion of the values that could be reasonably be attributed to the analyte. The expanded uncertainty represents an interval around the results where the unknown true value can be observed with a confidence level of 95%. The relative expanded uncertainties (%) are obtained by dividing

Table 2
Trueness, precision, accuracy and uncertainty ($n = 3$).

	Level	Folic acid		Level	Folic acid
Trueness			*Accuracy*		
	1	−0.87		1	[−5.71;3.97]
	2	0.36		2	[−5.40;6.13]
Relative bias (%)	3	−0.38	beta-expectation tolerance limits (%)	3	[−4.81;4.04]
	4	0.48		4	[−1.41;2.37]
	5	0.20		5	[−5.58;5.98]
Intra-assay precision			*Uncertainty*		
	1	0.64		1	3.49
	2	0.84		2	4.48
Repeatability (RSD%)	3	0.45	Relative expanded uncertainty (%)	3	3.19
	4	0.19		4	1.40
	5	0.16		5	4.20
Between assay precision					
	1	1.62			
	2	2.06			
Intermediate precision (RSD%)	3	1.47			
	4	0.62			
	5	1.90			

the corresponding expanded uncertainties with the corresponding concentrations. Results are shown in Table 2.

3.3.4. Recovery

The absolute recovery of folic acid was determined at the five concentration levels used to construct the accuracy profile. Following mean recoveries were obtained: concentration level 1 99.13%, concentration level 2 100.36%, concentration level 3 99.62%, concentration level 4 100.48% and concentration level 5 100.20%. All recoveries are within acceptable limits, indicating that the method is suited for the analysis of folic acid in pharmaceutical preparations.

3.3.5. Robustness

Robustness is a measure for the influence of small changes in the analytical procedure/parameters on the response.

The test was performed by a three-factor three-level full factorial design, using the flow, the column temperature and the percentage of formic acid in the aqueous phase as factors and the resolution between vitamin B2 and folic acid (critical pair) as response. The different levels were chosen based on the errors which are common during such an analysis. The levels for the flow were set at 0.25 ml/min (−1), 0.30 ml/min (0) and 0.35 ml/min (1), for the temperature at 39 °C (−1), 40 °C (0) and 41 °C (1) and for the percentage formic acid at 0.09% (−1), 0.1% (0) and 0.11% (1). All experiments were performed in random order.

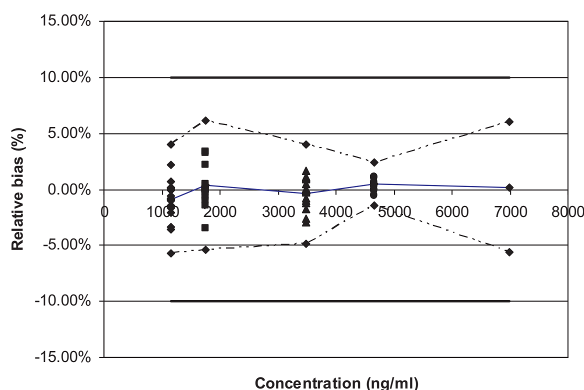

Fig. 3. Accuracy profile of folic acid. The plain line is the relative bias, the dashed lines are the β-expectation tolerance limits, the bold plain line is the acceptance limits (10%) and the dots represent the relative back-calculated concentrations, plotted with respect to their targeted concentration.

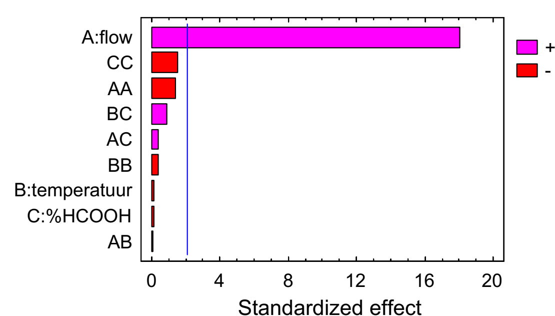

Fig. 4. Standardized Pareto chart for the resolution between folic acid and vitamin B_2.

The effects of the different factors were calculated and their significance at the 5% level was tested using an ANOVA analysis. Fig. 4 shows the standardized Pareto chart representing the significance of the factors on the resolution between folic acid and vitamin B2. From the ANOVA analysis it could be seen that the regression is significant with an R^2 of 95.12%. From the ANOVA table shown in Table 3 it could be seen that only the flow had a significant effect on the resolution between folic acid and vitamin B_2. It seems that the flow has a statistically significant effect on the retention in this UHPLC method. Also the ranges of the flow went from 0.25 ml/min to 0.35 ml/min, which is quite a large range for UHPLC. Even though the flow was considered to have a statistical significant effect, the resolution between folic acid and vitamin B_2 varied from 5.2 to 4.8 with increasing flow rate. Since two signals are considered to be baseline separated with a resolution of two, this will not influence the analysis of folic acid and the method can be considered as suited for purpose.

Table 3
Analysis of variance for the resolution of folic acid.

Factor	Sum of square	Degrees of freedom	Mean square	*F*-ratio	*P*-values
Flow (A)	0.61	1	0.61	325.90	0.0000
Temperature (B)	0.00	1	0.00	0.01	0.9144
% formic acid (C)	0.00	1	0.00	0.01	0.9144
AA	0.00	1	0.00	2.01	0.1746
AB	0.00	1	0.00	0.00	0.9475
AC	0.00	1	0.00	0.16	0.6936
BB	0.00	1	0.00	0.14	0.7102
BC	0.00	1	0.00	0.75	0.3973
CC	0.00	1	0.00	2.28	0.4190
Total error	0.03	17	0.00		

Table 4
Results for the analysis of a commercial sample with the UHPLC method and the reference method.

Day	HPLC	UHPLC
1	94.64%	93.10%
2	92.12%	92.36%
3	92.39%	92.93%
4	92.06%	93.02%
5	90.66%	92.03%
Grand mean	92.27%	92.69%

3.4. Comparison with the reference method

A commercial sample containing 5 mg of folic acid per capsule was analysed five times on five consecutive days and this with the developed UHPLC method and with the HPLC reference method. Standards and samples were prepared following the protocol described in Section 2.5.

Each day the sample was analysed in double with both methods. Table 4 shows the mean results obtained for both methods during the five days.

In a first step the variances of both methods were compared using a two-sided F-test at significance level $\alpha = 0.05$. The variance for the reference method (σ_A^2) was 0.569 and for the UHPLC method (σ_B^2) 0.144. The calculated F-statistic was 9.39 and since this is smaller that the critical value 9.60 ($\alpha = 0.05$, degrees of freedom 4 for both methods) the variances are considered equal and can be pooled.

The bias between both methods was evaluated using a paired t-test comparing the grand means of both methods. The calculated t-statistic was 0.47 and since this is smaller than the critical value 2.31 ($\alpha/2 = 0.025$, degrees of freedom 8) the differences between both grand means were considered insignificant.

Even if previous test shows an insignificant difference between the two methods the risk of adopting a method with an unacceptable bias can be reduced by performing an interval hypothesis test as described by Hartman et al. [20,23]. For the interval hypothesis test a bias of 3% was said to be acceptable. The interval calculated was [−0.939:1.569] which is well within the limits of 3%.

4. Conclusions

An ultra fast liquid chromatographic method was developed and validated for the qualitative and quantitative analysis of folic acid in pharmaceutical preparations. The validation was performed following the ISO17025 and proved that the method was suited for purpose and can be used in the routine analysis of folic acid in pharmaceutical preparations.

The method is a gradient method, using 0.1% formic acid in water (pH ∼2.6) as aqueous phase and acetonitril as organic phase. The gradient starts at a percentage of 98% of the formic acid solution and comes to a plateau of 80% 0.1% formic acid at 3 min. The flow rate is 0.3 ml/min and the detection wavelength 285 nm.

Comparison of the developed method with an HPLC method used in routine at our lab showed no statistically significant differences in the results obtained with both methods.

The developed method has some practical advantages compared to the HPLC method. UHPLC not only cuts run times with 50% (5 min in stead of 10 min) and reduces flow rates from 0.7 ml/min to 0.3 ml/min, which represents a significant gain in analysis times and solvent consumption, it also uses a much simpler aqueous phase to prepare, which leads to a decreased workload.

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