Journal of Chromatographic Science 2013;51:544–551
doi:10.1093/chromsci/bms170 Advance Access publication October 24, 2012

Article

Online Pre-Column Derivatization with Chromatographic Separation to Determine Folic Acid

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Received 4 April 2012; revised 31 July 2012

A simple, sensitive, and selective online pre-column derivatization high-performance liquid chromatographic method was developed and validated for the first time to determine trace levels of folic acid (FA). An oxidant cerium (IV) trihydroxyhydroperoxide packed reactor was used for pre-column oxidation and was combined by column switching with a C18 analytical column for sample enrichment and separation. The method was based on oxidative cleavage of FA into highly fluorescence products, 2-amino-4-hydroxypteridine-6-carboxaldehyde and the corresponding 2-amino-4-hydroxypteridine-6-carboxylic acid, during the flow of 0.04 M phosphate buffer (pH 3.5) containing the analyte through packed reactor at a flow rate of 0.2 mL/min and 40°C. The fluorescent products were enriched on the head of the analytical column for the final separation. The separation was performed at room temperature using a mobile phase consisting of phosphate buffer (0.04 M, pH 3.5) and acetonitrile (90:10, v/v). The eluents were monitored at emission and excitation wavelengths of 463 and 367 nm, respectively. The method showed excellent recovery, precision and accuracy with detection limits of 0.067 ng/mL from 500 μL of sample FA. The developed method was successfully applied to the determination of FA in pharmaceutical formulations and showed a recovery of 99.31% and a relative standard deviation of 1.72%.

Introduction

Folic acid (FA) {N[4-((2-amino-l,4-di-hydro-4-oxo-6-pteridinyl-methyl)amino)-benzoyl]-L-glutamic acid} (Figure 1), which occurs naturally in cereals, is essential to humans. FA plays a major role in the synthesis of red blood cells, in the formation of RNA and DNA and in the development of the tissues and brain of a fetus and the growth of a baby (1).

Many methods have been developed for the determination of FA, including spectrophotometry (2, 3), fluorimetry (4–7), chemiluminescence (8), enzyme assay (9), capillary electrophoresis (10–13) and flow injection analysis (14–17). High-performance liquid chromatography (HPLC) with ultraviolet (UV) detection has been widely used as the simplest method with the highest selectivity in the determination of FA in pharmaceutical formulations and biological fluids (18–25). The United States Pharmacopoeia for the determination of FA is based on extraction of the drug followed by HPLC with UV detection at 254 nm (26). Due to its higher sensitivity and selectivity, the measurement of very low FA is more precise in HPLC with fluorescence detection. Because of a lack of significant native fluorescence, offline pre-column derivatization has been chosen as a technique to increase the detectability of FA (27–32). However, lengthy sample derivatization was the most commonly reported limitation of these methods.

In recent years, more strict regulation related to the quality control of pharmaceuticals has led to increasing demands on the automation of the analytical assays conducted in appropriate control laboratories. Automated analyses promise a major reduction in manual laboratory working time, and correspondingly a significant cost reduction. Additionally, results of an automated method are less dependent on the experience of the analyst than those of a manual method. Thus, analytical methods are more easily transferred to subsidiary laboratories, and results tend to be more precise and reproducible.

Although many HPLC methods are available for FA determination, no automated pre-column-based technique has hitherto been reported that can determine the analyte in pharmaceutical preparations with high simplicity. One of the possibilities to facilitate and accelerate the analysis is to integrate the sample derivatization step directly into the flow injection analysis system coupled to HPLC. Therefore, this study was involved in a research effort aimed at expanding the automation by incorporating online oxidation and pre-concentration steps before the HPLC separation of FA using a column switching technique. The method was based on oxidative cleavage of FA during the flow of carrier stream containing the drug through cerium (IV) trihydroxyhydroperoxide (CTH) packed oxidant reactor to give highly fluorescent products, 2-amino-4-hydroxypteridine-6-carboxaldehyde and the corresponding 2-amino-4-hydroxypteridine-6-carboxylic acid (Figure 1). It has been reported that a CTH packed reactor was employed for the flow injection analysis of the methotrexate (33). The molecular structure of methotrexate, as shown in Figure 1, is very similar to that of FA; it differs only in that FA has a hydroxyl group in place of the 4-amino group on the pteridine ring and has no methyl group at the N^{10} position. This study demonstrated that supplanting the offline derivatization with automated oxidation using CTH as a packed reactor offered certain advantages over homogeneous reagent solutions. Reagent consumption was greatly decreased and the system was simplified with fewer junctions for the mixing of reagent, sample and carrier streams. Also, online pre-concentration before HPLC

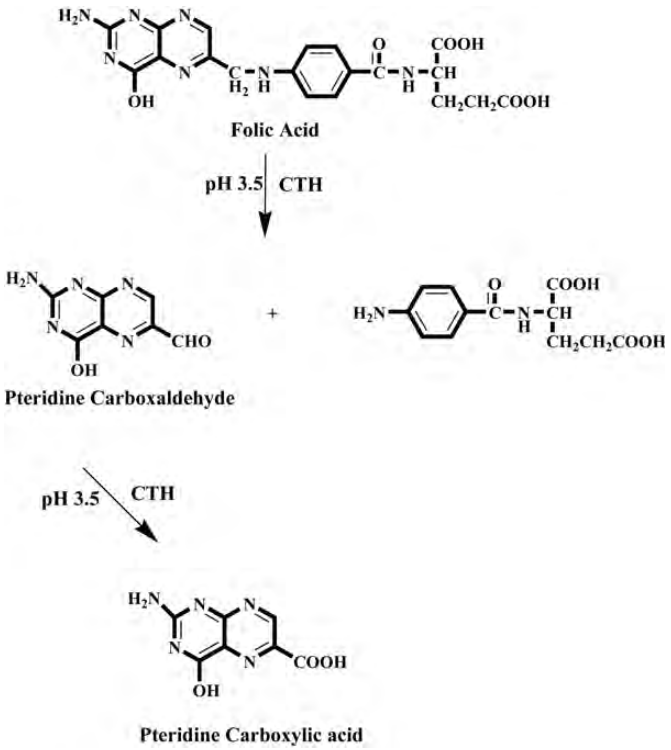

Figure 1. Structures of the investigated compounds.

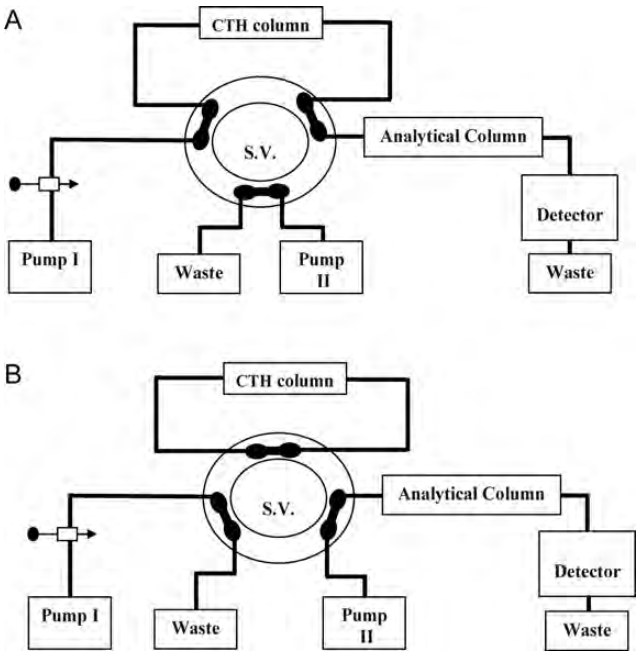

Figure 2. Schematic diagram of the online pre-column HPLC for the analysis of FA in pharmaceutical formulation: the system in initial position, ready for sample injection, derivatization and enrichment steps (A); the separation step, in which the CTH column is isolated from HPLC circulation (B) (S.V.: six-port switching valve).

separation can enhance the concentration detection limits of FA. The applicability of such an approach was demonstrated by determination of FA in pharmaceutical formulations. The combination of pre-column derivatization, enrichment and chromatographic separation with fluorescence detection appeared to be a viable approach for the rapid determination of FA in pharmaceutical formulation down to a level of 0.067 ng/mL.

Experimental

Instrumentation

The HPLC (Agilent Technologies, Palo Alto, CA) apparatus, illustrated in Figure 2, consisted of two solvent delivery pumps (Agilent 1100 Series Iso pump G1310A). One was used to deliver the carrier solution at a flow-rate of 0.2 mL/min and the other delivered the isocratic mobile phase at a flow rate of 1 mL/min. A model 7125 sample injection valve (500 μL) and a model 7010 flow direction switching valve were applied to load the sample onto the CTH-packed reactor, to facilitate oxidative cleavage of FA into highly fluorescence products and to control the flow direction switching and isocratic elution, respectively (Rheodyne, Berkeley, CA). This system was equipped with two columns; one was a short (50 × 7.5 mm i.d.) CTH oxidant column for oxidative cleavage of FA into highly fluorescent products and the other was a Thermo Scientific Hypersil ODS analytical column (100 × 4.0 mm i.d., 5 μm particle size; Thermo Scientific, West Palm Beach, FL). A fluorescence detector monitored the eluents (Agilent 1200 series, G1321A), set at an excitation wavelength of 367 nm and an emission wavelength of 463 nm. Data acquisition was performed on Agilent LC ChemStation software. The analytical column temperature was ambient, and that of the CTH-packed reactor was 40°C.

Materials and reagents

FA (99.81% purity) was obtained from Kyowa Hakki (Tokyo, Japan). The present method was applied to the determination of FA in its pharmaceutical formulations. FA tablet (Batch No. 940111) was manufactured by Mepaco Medifood Enshas (Sharkeya, Egypt). Each tablet was claimed to contain 5,000 μg of FA. The acetonitrile was HPLC grade (BDH, Poole, UK). Distilled water was used for the preparation of all reagents and solutions. Potassium dihydrogen phosphate, ortho-phosphoric acid, sodium hydroxide, hydrochloric acid, ethanol and isopropyl alcohol were analytical grade.

Preparation of CTH column

The CTH packing materials were suspended in isopropyl alcohol and degassed under vacuum with continuous stirring for 10 min. A stainless-steel cylinder (100 × 7.5 mm i.d.) was used as a reservoir for the CTH packing materials. This reservoir was connected to a short column (50 × 7.5 mm i.d.) and the suspended CTH supplied from the reservoir was packed into the column with the aid of an HPLC pump at flow rate of 5 mL/min, with ethanol as a purge solvent (10 min). Pumping continued until a constant pressure was reached. The cylinder was then disconnected and a mixture of ethanol and distilled water (1:1) was passed through the column at a flow rate of 1 mL/min for a further 10 min. The column was then

equilibrated with 0.04M phosphate buffer of pH 3.5 at a flow rate of 1 mL/min for 30 min.

Mobile phases

Two different mobile phases were employed in the assay procedure. One was 0.04 M phosphate buffer, pH 3.5, which was used as a carrier stream (MI) to deliver the sample to the CTH packed reactor in the oxidation step. The other was an isocratic solvent system (MII) consisting of acetonitrile and phosphate buffer (0.04 M, pH 3.5) (10:90, v/v) and used to elute the enriched fluorescent products from the head of the Thermo Scientific Hypersil ODS analytical column to the fluorescence detector for further separation. All solvents were freshly prepared on the day of use, filtered through 0.45 μm filters and degassed ultrasonically under vacuum.

General procedure

A 500 μL aliquot of FA sample was loaded into the injection valve and then injected into MI. The moving zone of FA passed through the CTH column at a flow rate of 0.2 mL/min (pump I). The oxidative cleavage of FA occured during the flow of MI containing the drug through the CTH column. Pre-concentration was performed by means of flow of the derivatized products from the oxidant column to the analytical column head. After 6 min, the valve was switched into position B (Figure 2). At this position, the MII could pass directly through the analytical column, where aldehydic and carboxylic fluorescent products were then separated. The flow rate was maintained at 1 mL/min and the fluorescence intensity of the eluting compounds was monitored at emission and excitation wavelengths of 463 and 367 nm, respectively. At 9 min after injection, the valve was switched into position A.

Standard solution and calibration

FA (2.5 mg) was transferred to a 200 mL conical flask with 150 mL distilled water. To promote the solution of FA, two drops of 1 M NaOH were added. Immediately after solvation, pH was adjusted to 7–8 with 0.1 M hydrochloric acid and the solution was quantitatively transferred to a 250 mL calibrated flask and completed to 250 mL with distilled water to produce a stock standard solution of 10 μg/mL. A known volume of the stock standard solution was diluted with the same solvent to obtain a concentration of 1 μg/mL FA (Solution A). The standard solutions for calibration were prepared daily by serial dilutions of appropriate volumes of Solution A to produce FA standard solutions in the concentration range of 1–40 ng/mL. An aliquot of 500 μL was analyzed for FA according to the proposed procedure. The stock standard solutions of FA were stored in a dark flask at −20°C and were found to be stable for at least one month. The standard solutions for calibration were freshly prepared and stored in a dark flask at 5°C during use.

Determination of FA in pharmaceutical formulations

A total of 20 tablets containing FA as the active ingredient were weighed and powdered. A portion of the powder equivalent to 2.5 mg of FA was accurately weighed and transferred to a 200 mL conical flask with 150 mL distilled water. Two drops of 1 M NaOH were added, and the resulting solution was sonicated for 30 min. The pH was adjusted to 7–8 with 0.1 M hydrochloric acid and the solution was quantitatively transferred to a 250 mL calibrated flask and completed to 250 mL with distilled water to produce a stock standard solution of 10 μg/mL; it was then filtered through a 0.45 μm membrane filter. The first portion of the filtrate was discarded and the remainder was used as a stock sample solution (Solution A, 10 μg/mL). A known volume of Solution A was quantitatively diluted with distilled water to obtain a concentration of 20 ng/mL of FA. An aliquot of 500 μL was analyzed for FA according to the proposed procedure. The sample solution was stored in a dark flask at 5°C during use.

Result and Discussion

The use of a packed reactor, in the described manifold, as an alternative to existing reagent solutions for the pre-column derivatization of FA, was dependent on optimization of the system to achieve the maximum detector response. On the basis of experimental results, the flow rate of the carrier stream and column dimensions were the key parameters, because they affected the extent of the residence time between the solid surface of CTH and the moving sample zone solutions. Also, the packed reactor temperature was found to affect the reaction completeness of FA with CTH. The detection wavelengths were chosen using the spectrum mode of the fluorescence detection with respect to the maximum sample signals. This mode enabled the determination of the optimum emission and excitation wavelengths in real conditions during measurement. The final measurement conditions were at emission and excitation wavelengths of 463 and 367 nm, respectively. The other parameters optimized were the pH, buffer concentration and the concentration of acetonitrile used as organic modifier in the elution solvent.

Effect of pH and concentration of carrier stream

Adjustment of the carrier stream pH was necessary to improve the reaction completeness between the analyte and the CTH packed reactor. The effect of this parameter was studied in the pH range 2.8–5 using buffer solutions from phosphate and acetate. Phosphate buffer gave the best performance as a carrier stream and was selected in all further experiments. As shown in Figure 3, the pH of the buffer solution severely affected the fluorescent derivatization of FA. The highest intensity was observed in the narrow range of pH 3.2–3.8 (Figure 3). At pH $<$ 3.0, a low signal response was observed, which might suggest the decomposition of the peroxy groups of the CTH materials; whereas at pH $>$ 3.8, the detector signals was decreased abruptly, probably due to the low CTH reactor efficiency, which drastically reduces the oxidative cleavage of FA into a highly fluorescent derivative. Accordingly, a phosphate buffer solution of pH 3.5 was selected as the optimum carrier stream.

The variation in the fluorescent intensity of FA with CTH packed oxidant was examined using phosphate buffer of concentrations varying from 0.02 to 0.1 M. The best analytical signals were verified within the concentration range of 0.02–0.05 M (Figure 4). When the concentration of buffer was

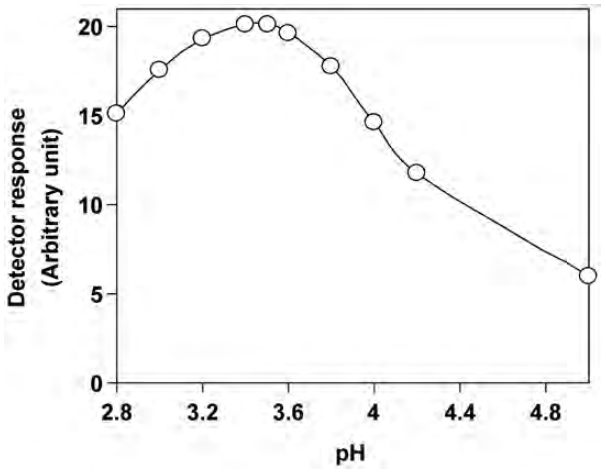

Figure 3. Effect of phosphate buffer pH on the reaction efficiency of the CTH-packed reactor with FA.

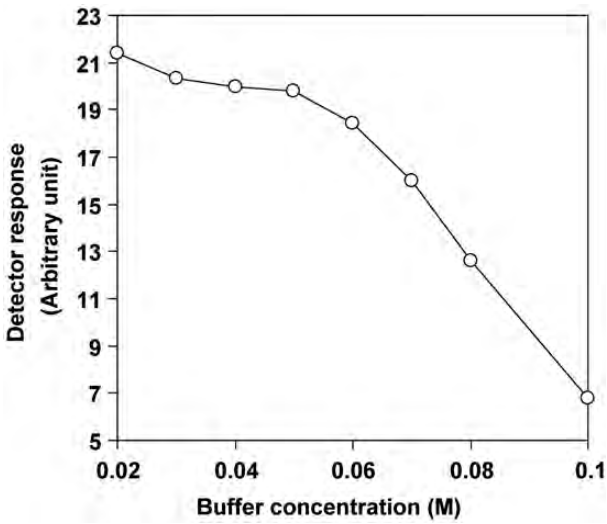

Figure 4. Effect of phosphate buffer concentration on the reaction efficiency of the CTH-packed reactor with FA.

increased, the detector response drastically decreased, probably due to the greater quenching effect on the fluorescence signal intensity. Although 0.02 M phosphate buffer showed a slight improvement of signal intensity, 0.04 M was chosen as a compromise between detector response and precision.

Effect of temperature

The packed reactor temperature has a critical effect on the reaction progress. The effect of this parameter on the derivatization of FA with CTH packed oxidant into highly fluorescent derivatives was investigated in the range of 25–65°C. Lower temperatures were inadequate, whereas elevating the temperature within the range of 25–65°C resulted in an increase in the reaction rate and subsequently, detector response (Figure 5). It was also observed that heating slightly above 45°C resulted in an increase in column pressure and showed a generally drastic effect on the CTH-packed oxidant life span. Considering the effective reaction temperature and the packed oxidant limitations, 40°C was selected as the optimum value, because under this condition, good sensitivity and reproducibility were achieved.

As a result, the optimal oxidation reaction efficiency of the CTH-packed reactor could be achieved by using 0.04 M phosphate buffer, pH 3.5 at a flow rate of 0.2 mL/min and 40°C.

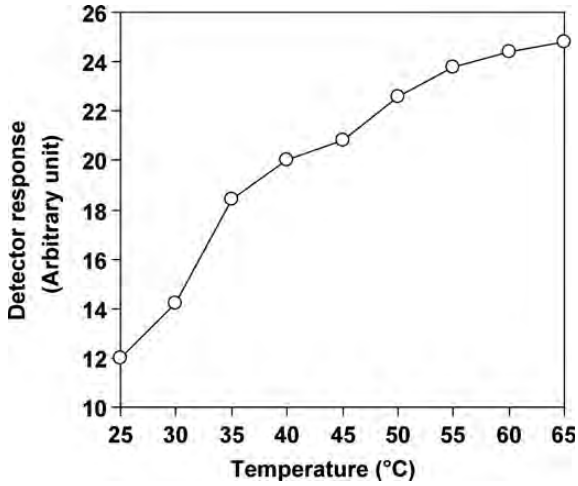

Figure 5. Effect of temperature on the reaction efficiency of the CTH-packed reactor with FA.

Effect of the flow rate

The reaction of the CTH column with FA was highly influenced by the flow rate of the carrier stream. The use of rapid analyte transport into and out of the packed reactor would be advantageous for the fast analysis; however, it was also essential that the flow rate of the sample stream not to be so rapid as to compromise the extent of the analyte in the packed reactor. The effect of the flow rate was checked over the range 0.2–0.8 mL/min. When the flow rate was reduced from 0.8 to 0.2 mL/min, a maximum increase in detector response of 2-amino-4-hydroxypteridine-6-carboxylic acid was observed (Figure 6). At the same time, the response ratio of the two products was also changed. Hence, it can be postulated that any decrease in the flow rate of the carrier stream will increase the residence time (reaction time) between the solid surface of the CTH and the moving zone of FA, whereas at higher flow rates, less fluorescent products can be produced and the recorded signal is decreased. The residence time between the sample zone containing FA and the solid-phase reactor is very important for the reaction to sufficiently proceed, and to achieve a substantial enhancement of the detector response. In the present work, a lower flow rate was justified for the determination of FA because peak enrichment could be achieved on the top of the analytical column (Hypersil ODS analytical column). As far as the oxidation reaction, 2-amino-4-hydroxypteridine-6-carboxaldehyde was converted into the corresponding 2,4-diaminopteridine-6-carboxylic acid. Thus, it can be deduced that the signal of 2-amino-4-hydroxypteridine-6-carboxylic acid became more predominant when a lower flow rate was employed. The fluorescent products could be accumulated on the top of the analytical column with a zone width, almost independent on the

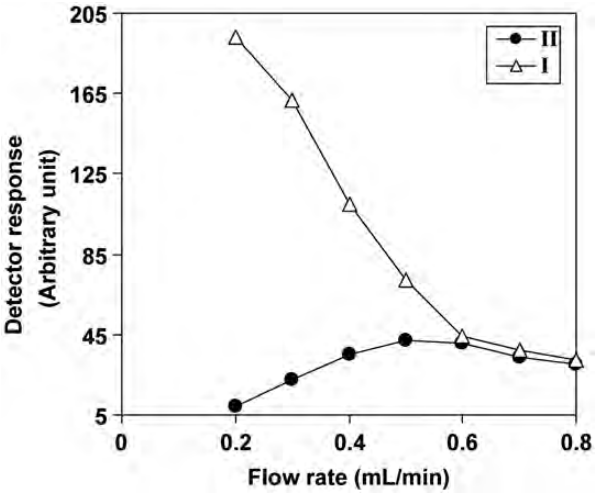

Figure 6. Effect of flow rate of the carrier stream on the detector response after oxidative cleavage of FA with CTH and fluorimetric detection (20 ng/mL). Peaks: I, 2-amino-4-hydroxypteridine-6-carboxylic acid; II, 2-amino-4-hydroxypteridine-6-carboxaldehyde.

flow rate. A compromise between analytical signal and sample frequency was established by choosing a working flow rate of 0.2 mL/min.

Column dimensions

The residence time between the sample zone containing FA and the CTH-packed reactor was very important for the reaction to proceed sufficiently. In the preliminary experiments, two packed reactors with different dimensions were examined to obtain maximum detector response. The influence of the packed reactor diameter on the sensitivity was studied at 4.6 and 7.5 mm (i.d.) using a column length of 20 mm. When the system manifold was equipped with a wider packed reactor (7.5 mm i.d.), the sample dispersion was significantly increased. Because a wider column can increase the residence time for the reaction to complete, there is an increase in a peak signal due to dispersion of the sample zone over a larger area of the solid reactor. Thus, it can be deduced that the effect of dispersion became more influential when a wider packed reactor was employed. A diameter of 7.5 mm (i.d.) was chosen to enhance the dispersion and to maintain an efficient residence time. The positive effect of the packed reactor on sensitivity was also observed by increasing the column length up to 50 mm. This can be explained by the fact that an increase in length gives a longer residence time and subsequently increases the peak area of FA. The previous set of experiments led to the following results: (i) the longest columns were suitable, as detector response was increased; (ii) the i.d. was the predominant parameter influencing sample dispersion and subsequently, the residence time. A packed column reactor of 7.5 mm (i.d.) and 50 mm length was therefore chosen.

Effect of sample volume

In an effort to push the concentration detection limit to a lower level, the analytical column head was used to pre-concentrate FA by loading a large sample volume of FA to the packed CTH reactor. The design of a switching valve containing a packed reactor for online pre-column derivatization and sample enrichment was described (Figure 2A). Samples were loaded onto the CTH packed reactor with a carrier mobile phase (MI) and pump I, while pre-concentration was performed by means of the flow of the derivatization products from the packed reactor to the analytical column head. Different injection volumes (50–600 μL) were tested to introduce decreasing concentrations of FA. The efficiency of the enrichment for FA was evaluated on the basis of the linearity of calibration graphs constructed over sample volumes (50–600 μL, at 50 μL intervals). It was found that the CTH-packed oxidant could tolerate large volumes of FA standard solution and the linear relationship between the peak area and the injected volumes was observed over the range of 50–500 μL sample volume. The regression equation for the influence of the sample volume on the analytical signal in this range was $Y = 0.7043V + 2.9149$ ($r = 0.9992$), where Y is the peak area and V denotes the loaded volume in μL of FA. If too large a sample is used (more than 500 μL), then the linearity of FA between the peak area and concentration will be disturbed, because a long residence time was found to be necessary to achieve reproducible results when using large volumes of FA samples. Accordingly, a sample volume of 500 μL FA sample was selected as a compromise between the sensitivity and accuracy. The pre-concentration was effective for the proposed method, achieving a detection limit of 0.067 ng/mL of FA for a sample volume of 500 μL with fluorescence detection.

Optimization of the chromatographic system

During the initial study of the pre-column derivatization using a packed reactor, it was noted that the CTH column needed a long equilibration time with 0.04 M phosphate buffer, pH 3.5 aqueous carrier stream (MI) after passing any acetonitrile containing mobile phase (MII) through it. To eliminate such a long equilibration time, the online pre-column derivatization system was set up with a column-switching technique and two pumps to independently deliver MI and MII.

The CTH derivatizing agent reacts with FA to form highly fluorescent 2-amino-4-hydroxypteridine-6-carboxaldehyde and the corresponding 2-amino-4-hydroxypteridine-6-carboxylic acid derivatives, which can be pre-concentrated and separated on a reversed-phase column. The optimization procedure was continued by changing the pH of the analytical mobile phase (MII) and by changing in the ratios of acetonitrile and phosphate buffer. Variation in the pH of the mobile phase in the range 2.0–6 weakly affected the retention behavior of the fluorescence products. Variation of the acetonitrile concentration strongly affected retention behaviors and band broadening of the fluorescence products. Using a short Thermo Scientific Hypersil ODS analytical column (100 × 4.0 mm, 5 μm), a mobile phase of acetonitrile and phosphate buffer (0.04 M, pH 3.5) (10:90 v/v) was found to give acceptable separation in a short time (Figure 7). By these means, faster separation was possible and the productivity of chromatographic processes was increased.

Validation

Linearity

The calibration curve for FA was constructed with seven concentrations (simultaneously prepared) ranging from 1 to

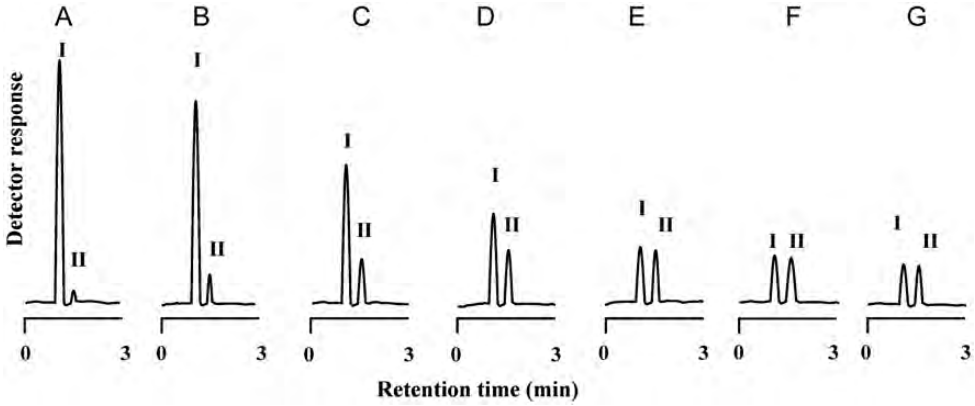

Figure 7. Chromatograms obtained after oxidative cleavage of FA with CTH and fluorimetric detection (20 ng/mL). Peaks: I, 2-amino-4-hydroxypteridine-6-carboxylic acid; II, 2-amino-4-hydroxypteridine-6-carboxaldehyde. Flow rates: 0.2 mL/min (A); 0.3 mL/min (B); 0.4 mL/min (C); 0.5 mL/min (D); 0.6 mL/min (E); 0.7 mL/min (F); 0.8 mL/min (G).

Table I
Characteristic Parameters for the Regression Equations of the Proposed HPLC Method*

Parameters	FA
Calibration range (ng/mL)	1–40
LOD (ng/mL)	0.067
LOQ (ng/mL)	0.225
Slope (*b*)	1.4113
Standard error of the slope	0.0193
Intercept (*a*)	0.5273
Standard error of the intercept	0.3824
Correlation coefficient (r^2)	0.9993

*Note: $Y = a + bC$, where C is the concentration of FA in ng/mL and Y is the peak area.

Table II
Precision and Accuracy Validation of FA

	Concentration (ng/mL)	Recovery (%) ± SD	RSD (%)	Mean RE (%)
Intra-assay*	5	99.28 ± 0.17	0.17	0.72
	10	99.32 ± 0.25	0.25	0.68
	20	99.37 ± 0.31	0.31	0.63
	30	99.26 ± 0.34	0.34	0.74
Inter-assay*	5	99.22 ± 0.19	0.19	0.78
	10	99.36 ± 0.28	0.28	0.64
	20	99.35 ± 0.34	0.34	0.65
	30	99.22 ± 0.37	0.37	0.78

*Average of five determinations.

40 ng/mL. A calibration curve was constructed by plotting the measured peak area versus concentration. Each concentration was repeated three times; this approach provided information on the variation in peak area values between samples of the same concentration. The linearity of the calibration graph was validated by the high value of the correlation coefficient (more than 0.999). The equation for the best-fit straight line was determined by the following linear regression analysis: $Y = a + bC$, where Y is the peak area and C denotes the concentration in ng/mL of FA. Characteristic parameters of the linear calibration curve are shown in Table I.

Limits of detection and quantification

The limit of detection (LOD), defined as the lowest concentration of FA that can be clearly detected above the base line signal, is estimated as three times the signal-to-noise ratio (34). The LOD was determined ($n = 6$) by injection of FA samples in decreasing concentrations. The LOD was found to be 0.067 ng/mL. The limit of quantification (LOQ) is often defined as 10 times the signal-to-noise ratio (34). The LOQ was determined ($n = 6$) by injection of the FA samples in decreasing concentrations. The precision was calculated for each concentration. The LOQ was then calculated as the concentration at which the precision was less than 15%, and was found to be 0.225 ng/mL. The LOD and LOQ values are reported in Table I.

Precision and accuracy

The precision of the analytical procedure was determined for both intra-day and inter-day variations and expressed as the relative standard deviation (RSD) (11) and the relative error (RE) (34) of the mean measured concentration. Repeatability (intra-day RSD, $n = 5$) was excellent, in the range of 0.17 to 0.34%, and the mean RE ranged from 0.63 to 0.74%. Reproducibility (inter-day RSD, $n = 5$) was in the range of 0.19 to 0.37% and the mean RE ranged from 0.64 to 0.78%. Repeatability and reproducibility of FA samples with high and low concentration levels were below 1%, indicating a reliable measurement using the proposed method (Table II). RE was evaluated by back-calculation and expressed as the percent deviation between concentration added and concentration found according to the following:

$$RE = [(\text{concentration added} - \text{concentration found}) / \text{concentration added}] \times 100$$

Column lifetime

The column lifetime, in terms of its ability to quantitatively derivatize FA to give highly fluorescent products, was investigated as a function of the volume of samples (20 ng/mL, FA) injected onto the CTH-packed reactor. It was found that this solid reactor could successfully be used for loading 100 mL samples. Increasing the injection volumes above this level led to a decrease in sensitivity and an increase of the column

Table III
Determination of FA in Commercial Tablets by the Proposed and Official Methods

Drug formulation	Mean found* (± RSD)	
	Proposed method	Official method
Tablets	19.828 ± 1.66	19.912 ± 1.20
t-test	0.39	(2.30)†
F-test	2.02	(6.38)†
Recovery‡ (%, ± RSD)	99.31 ± 1.72	

*Mean and SD for five determinations.
†Theoretical values for *t*- and *F*-tests.
‡For standard addition of 50% of the nominal content.

backpressure with final clogging. Accordingly, a CTH column should be renewed when it shows excessive backpressure or lower efficiency. Repetitive injections of 500 μL of FA (20 ng/mL), five times, each under continuous operation for 8 h every day over a period of 14 days, was conducted. It was found that the CTH column retained 99.15% of its efficiency over this period, after which a gradual pressure build-up at the head of the column was observed. The day to-day relative standard deviation for FA was found to be less than 4%.

Application

The proposed pre-column derivatization method was applied for the determination of FA in its commercial formulations together with the official United States Pharmacopeia (USP) method (26). As indicated, the assay results obtained by the proposed method were in accordance with those obtained by the official method. The accuracy and precision of the developed method were further judged by applying *t*- and *F*-tests at a 95% confidence level. The experimental *t*- and *F*-values did not exceed the theoretical values, which support the similar accuracy and precision of the proposed and official USP methods (Table III). The accuracy of the present method was also checked by the standard addition method, which was applied by adding drug standard to previously analyzed tablets. The accuracy shows that the derivatization procedure developed for the determination of FA can be considered to be accurate within the investigated concentration range (Table III). The mean value is very close to the theoretical concentration, showing method percent recovery of 99.31 and RSD of 1.72%. These results indicate that the effects of the common additives and ingredients of the pharmaceutical formulations do not interfere with the determination of FA.

Interference studies

To examine the selectivity of the proposed method, the effect of common excipients normally used in pharmaceutical formulations was studied. Solutions containing FA (20 ng/mL) were prepared in the presence of more than 100 common additives such as lactose, glucose, fructose, dextrin, magnesium stearate, xanthan gum, corn starch and sucrose. The undissolved materials were filtered off before injection. No significant changes were observed on the results, and recoveries in the range of 99.22–99.36% were obtained in all cases.

Robustness

To determine robustness of the proposed method, experimental conditions such as flow rate of carrier stream and mobile phase, pH, organic content of the mobile phase, and packed reactor temperature were purposely altered and the detector responses were evaluated. Variations of each parameter by ± 2% did not have a significant effect on the detector response.

Conclusion

In this study, the first online pre-column derivatization using a CTH-packed reactor followed by pre-concentration and separation with HPLC has been successfully applied to determine FA. The method was a powerful analytical technique that showed excellent sensitivity and sufficient accuracy, and required relatively simple and inexpensive instrumentation. These advantages made the proposed method an attractive procedure for the determination of FA at very low concentrations, down to a level of 0.067 ng/mL. The applicability of this method was evaluated by the determination of FA in pharmaceutical formulations. Common excipients used as additives in pharmaceutical preparations did not interfere.

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