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Article

Development of a Procedure Based on Chemiluminescence and Multicommutation Approach for the Determination of Folic Acid in Pharmaceuticals

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This paper presents an analytical procedure for the determination of folic acid employing commutation flow analysis process and detection by chemiluminescence. The procedure is based on the reaction of hexacyanoferrate(III) with folic acid, followed of luminol oxidizing reaction in alkaline medium, resulting in emission of radiation at 425 nm. After optimization the experimental variables, the proposed procedure afforded the following useful features. A linear response ranging from 0.1 to 1.00 mg mL^{-1} folic acid ($R^2 = 0.993$), a detection limit (3σ criterion) 0.046 mg mL^{-1}, a sampling rate of 156 determination *per* hour, a relative standard deviation less than 0.5% (n = 6) for a 0.6 mg mL^{-1} folic acid standard solution and a waste generation of 0.86 mL *per* determination.

Keywords: folic acid, multicommuted flow analysis, chemiluminescence, green chemistry

Introduction

Folic acid (*N*-[*p*{[(2-amino-4-hydroxy-6-pteridinyl) methyl]amino}benzoyl]-*L*-glutamic acid, is a water soluble vitamin belonging to the B complex (vitamin B9 or vitamin M),[1,2] which is produced by plants (green leaves, algae) and micro-organisms.[3-6] Several papers has been pointed out that folic acid contribute to the formation of red blood cells, being also identified as an anti-anemia and growth factor.[3,7,8] This compound participates in the synthesis of DNA bases and chains, being required for the formation of new cells, mainly in condition of rapid cell division and growth, including pregnancy and infancy.[9] The synthesis of the heme group of hemoglobin has also the participation of folic acid, therefore deficiency of folic acid causes several diseases, including megaloblastic anemia, bone marrow or fetal diseases (spina bifida, neural tube defects).[8-11] Cancer, Alzheimer's disease and cardiovascular disease in adults have been related with folic acid deficiency.[1,8-10] Deficiency in folic acid can causes gigantocytic anemia associated with leukopenia, devolution of mentality, psychosis, etc. Folate deficiency is believed to be the most common vitamin deficiency in the world due to food processing, food selection, and intestinal disorders.[10]

Clinical assays involving supplementation of folic acid during the pre-conception, observed a decrease in the risk of neural tube defects (NTDs).[12,13] For this reason, the United States (US) Public Health Services and other agencies recommend that all women of reproductive age should consume daily 0.40 mg folic acid supplement.[14,15] The US and other countries have mandated folic acid fortification of the food supply, specifically for the purposes of reducing the prevalence of NTDs.[14,15] The Brazilian Agency for Public Health Surveillance (ANVISA) required the mandatory fortification of wheat and corn flours with folic acid at a concentration of 1.50 mg g^{-1}.[12] In view of the benefits for health, preventing a range of disorders, a consumption of 0.4 mg of synthetic folic acid has been recommended,[15] including fortified foods or vitamin tablets.[6,8-10]

In view of the beneficial effect for health, the consumption of folic acid as pharmaceuticals become usual, whereby the availability of analytical method for its determination, become an essential requirement to allow the product valuation. The determination of folic acid in pharmaceuticals has been performed employing analytical procedures based on high-performance liquid chromatography (HPLC),[12,16] spectrophotometry,[1,2,13] capillary electrophoresis,[3,17] and chemiluminescence.[5,7,9]

Among the cited works, those based on chemiluminescence employed the simpler detection equipments, which do not

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use either a radiation source or an optical device, such as a light monochromator. Nevertheless, the detection equipment employed photomultiplier as an electromagnetic radiation sensor, which is higher sensitivity than a photodiode, but is also more expensive. The amount of folic acid in pharmaceuticals, allows that solutions can be prepared at appropriate concentration, allowing that the analyte determination would be formed, employing a very simple radiation detection instrument based on a photodiode.

Nowadays, there is a concern focused on the environmental sustainability of the analytical procedures, which would follow the green analytical chemistry (GAC) guidelines.[18,19] Among the GAC requisites, reduction of waste generation has received great attention because of its direct impact on the environment. This requisite has been properly accomplished, developing the analytical procedures based on the processes named as sequential injection analysis (SIA)[20,21] and multicommuted flow analysis (MCFA).[22,23]

The MCFA process, when implemented employing solenoid mini-pump for fluid propulsion, afforded facilities to meet the GAC requisite, concerning to reduction of waste generation.[18,19] This feature is exploited in this work to develop an automated analytical procedure environmentally friendly for the determination of folic acid in pharmaceuticals. The detection by chemiluminescence is performed employing a homemade luminometer. The procedure is based on the reaction of folic acid with hexacyanoferrate(III), wherein the oxidant consumption occurs. The hexacyanoferrate(III) remaining oxidizes luminol, generating radiation in the visible range of the electromagnetic spectrum (λ_{max} ca. 425 nm).[5,6]

Experimental

Reagents and solutions

All solutions were prepared using purified water with a specific resistivity of 18.2 mΩ cm^{-1} at 25 °C . The reagents were of analytical grade. A 2.0 mol L^{-1} sodium hydroxide stock solution was prepared dissolving the solid (Merck 99%) in water. Working hydroxide solutions were prepared by dilution with water. A 5×10^{-2} mol L^{-1} luminol stock solution was prepared by dissolving 221.5 mg of solid (Sigma 97%) in 25 mL of a sodium hydroxide solution (0.1 mol L^{-1}). This solution was stored in amber vial, covered with aluminum foil and kept refrigerated at 8 °C. The working solution with a concentration of 3.0 mmol L^{-1} was prepared daily by dilution with a 0.3 mol L^{-1} sodium hydroxide solution. A 0.1 mol L^{-1} potassium hexacyanoferrate(III) stock solution was prepared by dissolving 0.823 g of solid (Sigma 97%) 25 mL of water. Working solutions with concentrations ranging from 0.6 to 8.0 mmol L^{-1} were prepared by dilution with water from the stock solution. A 5.0 mg mL^{-1} folic acid stock solution was prepared by dissolving 250 mg of solid (Sigma 97%) using 3 mL of a 0.1 mol L^{-1} sodium hydroxide solution. After dissolution the volume was made up to 50 mL with water. Working standard solutions within the concentration range 0.1 to 2.0 mg mL^{-1} were prepared daily by dilution with water.

Sample preparation

A mass equivalent to a tablet was transferred to a 25 mL volumetric flask, which previously was loaded with 20 mL of water plus 1 mL of a 0.1 mol L^{-1} NaOH solution. After stirring for 5 min to promote dissolution, the solution was filtered and the volume was completed to 25 mL with water.

Standard solutions to study if excipients, usually present in the pharmaceutical of interest, cause or not interference, were prepared to comprise 0.5 mg mL^{-1} folic acid with and without the potential interferings. The compounds assayed were polyvinylpyrrolidone, starch, caffeine, cellulose, lactose, ethanol, methanol and ascorbic acid.

Apparatus and accessories

The equipment setup comprised a microcomputer, furnished with PCL711S interface card (Advantech Corp.) and running a software wrote in Quick BASIC 4.5; solenoid mini-pumps, two 120SP1220 and two 120SP1210 (BioChem Valve lnc.); one digital interface based on the integrated circuit ULN2803, used to drive the solenoid mini-pumps, assembled as described elsewhere;[24] a regulated power supply of 12 V and electric current intensity of 2 A, used to feed the solenoid mini-pumps; a regulated power supply of –12.0 V and +12.0 V and electric current intensity of 0.5 A, used to feed the luminometer. The accessories consisted of three OP07 operational amplifier; two photodiodes S1227-1010BR (Hamamatsu), two meters of polyethylene tube to construct flow lines and reaction coils; one metallic box with dimension of 25 cm wide, 20 cm deep and 10 cm height, used to accommodate the luminometer; resistors and capacitors used construct the luminometer as indicate in Figure 1.

Luminometer description

The luminometer was designed using two silicon photodiodes with a 100 mm^2 photo-sensible surface, which were wired to the operation amplifiers, as shown in Figure 1.

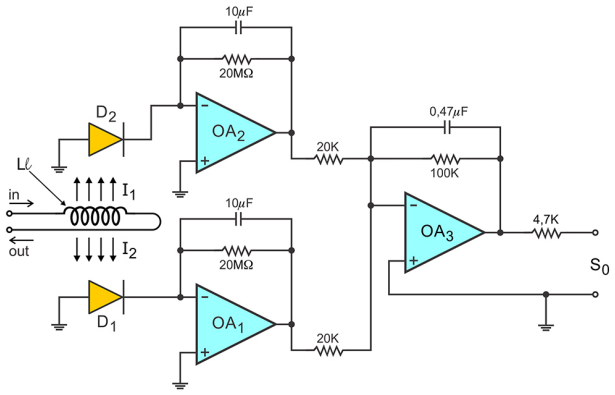

Figure 1. Diagram of the luminometer. D_1 and D_2 = photodiodes; OA_1, OA_2 and OA_3 = operational amplifiers OP07; S_0 = generated signal (mV); I_1 and I_2 = radiation beams emanated from the reaction coil; Ll = chemiluminescence reaction coil.

The operational amplifiers OA_1 and OA_2 are configured to form the signal transduction unit, which convert the electromagnetic radiation to an electric signal. The third operational amplifier (OA_3) is wired as a signals summing circuit and amplifier. In this configuration, the output signal is the sum of input signals multiplied by five. The luminometer, including the reactor coil, where radiation was emanate due to luminol oxidation, was accommodate into a metallic box to prevent external radiation.

Description of the flow system module

The analytical procedure is based on reaction of folic acid with hexacyanoferrate(III). The remaining hexacyanoferrate(III) reacts with luminol in alkaline medium, causing its oxidation, resulting in emission of radiation.[5,6] The flow analysis module was designed to accomplish the requisites for reaction development and its diagram is shown in Figure 2.

The solenoid mini-pumps P_1, P_2, P_3 and P_4 used for fluid propulsion, works following an on/off switching pattern, thus when an on/off action is applied to the solenoid mini-pump, an aliquot of the fluid is delivered. Considering this feature, the control software was designed to drive the solenoid mini-pumps following the time switching pattern showed in the bottom of Figure 2. When the control software was run, the microcomputer sent through the output port of the PCL11S interface card, a set of control signal in order to switch on/off the solenoid mini-pumps, following the time switching patter depicted in the switching time diagram.

The control signals were generated at the TTL pattern, and the integrate circuit ULN2803 convert them to 12 V, required do feed the solenoid mini-pumps. As depicted on the time switching diagram, an analytical run comprised three steps labeled as St_1, St_2 and St_3. As we can see in Figure 2, the sampling step consist of three sampling cycles,

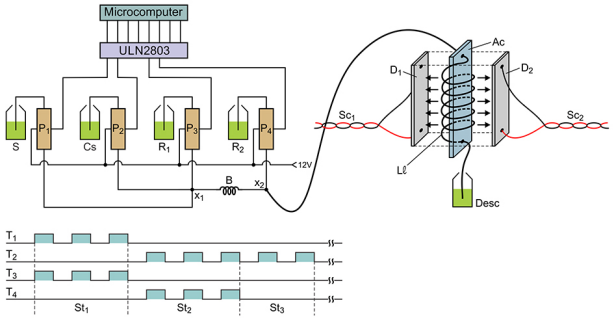

Figure 2. Diagram of the flow system module. S = sample or standard solution; Cs = carrier fluid; R_1 = hexacyanoferrate(III) solution; R_2 = luminol solution; P_1, P_2, P_3 and P_4 = solenoid mini-pumps; x_1 e x_2 = joint flow lines machined in acrylic; B = reactor coil of polyethylene tube, 50 cm long and 0.8 mm inner diameter; D_1 and D_2 = photodiodes; Ac = acrylic plate, 2.0 mm thickness and 6.0 mm wide; Ll = luminescent reactor coil, polyethylene tube, 20 cm long and 0.8 mm inner diameter; Sc_1 and Sc_2 = shielded cables to be connected to the input of the operational amplifiers (see Figure 1); w = waste. T_1, T_2, T_3 and T_4 = switching time diagram of the solenoid mini-pumps P_1, P_2, P_3 and P_4, respectively. St_1, St_2 and St_3 = sampling step, luminol inserting step and washing step, respectively. The shadow surface beneath of the time lines indicates that corresponding device was switched on.

which was carried out by switching on/off at the same time the mini-pumps P_1 and P_3. Under this condition, the reaction coil (B) was loaded with a mix comprising sample and hexacyanoferrate(III) solution. The number of sampling cycles can be increased in order to improve sensitivity. In the second step (St_2), mini-pumps P_2 and P_4 were switched on/off at the same time. When this happen, the mix comprising sample and hexacyanoferrate(III) solution, displaced by the carrier fluid (Cs), merged into the joint device (x_2) with aliquots of luminol solution. In the first step (St_1), the hexacyanoferrate(III) oxidized folic acid, the remaining of this reagent oxidized luminol into the chemiluminescence reactor coil (Cl), resulting in emission of radiation.

The chemiluminescence reactor Cl consists of a polyethylene tube wrapped on an acrylic plate, 6 mm width and 2 mm thickness. This assembling was chosen to obtain a reactor coil with a flattened shape, thus facilitating its installation between the two photodiodes, as shown in Figure 2. This arrangement allows that the major part of the radiation emanated from the reactor (Cl) was collected by the photodiodes (D_1 and D_2). The magnitude of the generated signal presents a linear relationship with intensity of the radiation generated when luminol was oxidized by the hexacyanoferrate(III) ions.

Results and Discussion

General comments

The analytical procedure was based on the reaction of folic acid with hexacyanoferrate(III) and the remaining

oxidant reacts with luminol in alkaline medium, resulting in emission of electromagnetic radiation. The chemical process occurs as shown below:[5]

Hexacyanoferrate(III) + folic acid $\xrightarrow{OH^-}$ hexacyanoferrate(II) + pteroic acid

Hexacyanoferrate(III) + luminol $\xrightarrow{OH^-}$ aminophthalate + $h\nu$ (λ_{max} = 425 nm)

where h = Planck constant and ν = frequency in Hz.

Once the pteroic acid does not oxidizes luminol, the radiation generated is in function of the concentration of the remaining hexacyanoferrate(III). Previous assays were performed to evaluate the response of the proposed luminometer. The assays were carried out employing the flow system depicted in Figure 2, and using solutions of luminol (3.0 mmol L^{-1}) in a 0.4 mol L^{-1} NaOH medium, folic acid (2.0 mmol L^{-1}) and potassium hexacyanoferrate(III) (0.6 mmol L^{-1}). The control software was settled to perform the steps St_1 and St_2, applying 10 and 12 sampling cycles for folic acid and luminol solution, respectively. The records depicted in Figure 3, shows that radiation inhibition occurred when hexacyanoferrate(III) and folic acid solutions were mixed prior to add luminol solution to the reacting medium.

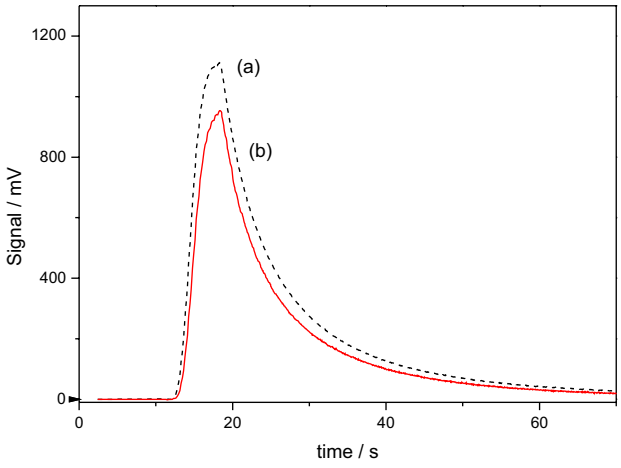

Figure 3. Records of the luminometer readout. Curves (a) and (b) are signals generated using a blank solution and a 0.6 mg mL^{-1} folic acid solution, respectively.

Effect sample and reagent solution volumes

In flow analysis process, the volume of sample plays an important role on the magnitude of the signals, which is generated as a function of the analyte concentration. Because of this, the volume of sample aliquot was the first variable studied, which was done by varying the number of sampling cycles (St_1, Figure 2) from 4 to 14 and maintaining 14 for luminol inserting cycles (St_2, Figure 2). Similar experiment was carried out maintaining 12 sampling cycles (St_1) and varying the number luminol inserting cycles (St_2). These assays were carried out using a blank solution instead of the folic acid solution. Taking the maximum values of the measurements, we derived the curves shown in Figure 4.

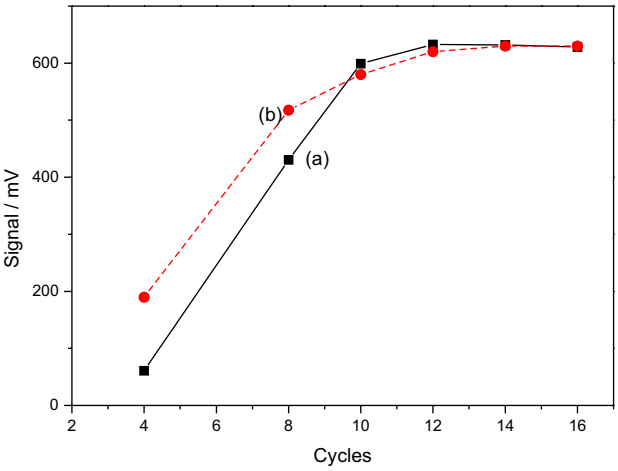

Figure 4. Effect of the sampling cycles. Curves (a) and (b) correspond to the variation of sample and luminol solution inserting cycles, respectively, achieved using a 1.0 mmol L^{-1} luminol solution prepared in a 0.4 mol L^{-1} NaOH medium and a 20 mmol L^{-1} hexacyanoferrate(III) solution.

Analyzing these curves, we observe a significant increase in the signal up to 10 sampling cycles, tending to a constant value for higher values. Similar effect also occurred while the number luminol inserting cycles varied from 4 up to 12. Based on these results, we selected 10 sampling cycles for folic acid and hexacyanoferrate(III) solutions and 12 inserting cycles for luminol and carrier solutions (St_1, St_2, Figure 2). The mini-pumps P_1 and P_2 delivered a volume of 20 µL *per* stroke, while P_3 and P_4 delivered 10 µL.

Under the settled operational condition, when performing the sampling step (St_1), the reactor coil (B) was load with a mix consisting of 200 µL of folic standard solution and 100 µL of hexacyanoferrate(III) solution, thus comprising a total volume of 300 µL, which was higher than the volume of the reactor B (250 µL). Owing to the generated signals shows a tendency to a constant value. The curve related with luminol inserting cycles has a similar behavior, when the sample zone displaced through the luminescent reactor (Ll) was higher than 360 µL.

Effect of the reagents solution concentrations

Intending to find the appropriated concentration of the $K_3[Fe(CN)_6]$ solution, assays were performed varying

the concentration of the oxidant solution, maintaining the luminol concentration used before and using a set of folic acid with concentration ranging from 0.1 to 1.0 mg mL^{-1}, yielding the results shown in Table 1.

Table 1. Effect of the oxidizing solution concentration

$[K_3[Fe(CN)_6]]$ / (mmol L^{-1})	Linear equation (n = 6)	R^2
0.8	y= –149.41[AF]^a + 660.37	0.999
1.0	y= –176.28 [AF]^a + 752.09	0.986
2.0	y= –200.08[AF]^a + 1007.70	0.996
3.0	y= –220.57[AF]^a + 1142.50	0.994

^{a}Acid folic concentration (mg mL^{-1}).

Analyzing the results showed in Table 1, we observe an increase of slope with the concentration of the oxidizing solution, whereas a decrease in linearity also occurred. Based on these results, the 2.0 mmol L^{-1} solution concentration was selected, considering as an acceptable compromise between sensitivity and the linearity.

The luminol oxidation to produce electromagnetic radiation occurs in alkaline medium, so the results previously commented were achieved using a luminol solution prepared in a 0.4 mol L^{-1} NaOH medium. Aiming to find the best alkaline condition for radiation emission, a set of assays was carried out varying the NaOH concentration, yielding results shown in Figure 5.

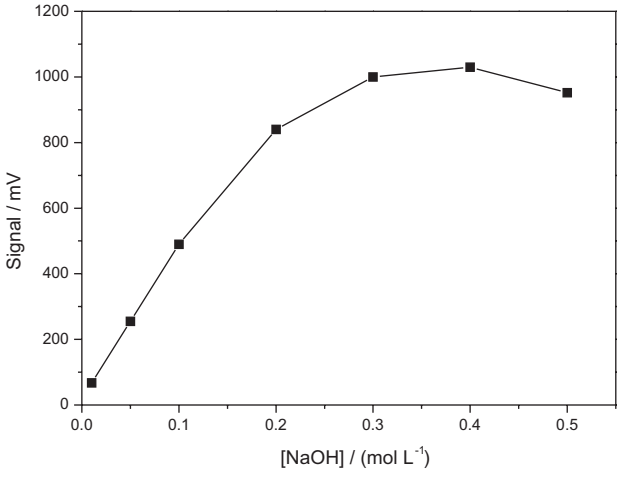

Figure 5. Effect of the sodium hydroxide concentration. Results obtained processing a blank solution. Other parameters as described in the previous sections.

Analyzing the curve shown in Figure 5, we observe an increase in signal with the sodium hydroxide concentration up to 0.3 mol L^{-1}, presenting decrease for concentration higher than 0.4 mol L^{-1}. Base on this result, the 0.3 mol L^{-1} concentration was chosen.

The assays previously described, were implemented in order to find the appropriate values of the experimental variables and the selected values are summarized in Table 2.

Table 2. Assayed parameters and selected values

Parameter studied	Range	Selected
$K_3[Fe(CN)_6]$ / (mmol L^{-1})	0.8-3.0	2.0
[NaOH] / (mol L^{-1})	0.01-0.5	0.3
Sampling cycles (P_1 + P_3)	4-16	10
Luminol inserting cycles (P_2 + P_4)	4-16	12

The signals summing effect

The luminometer represented by the diagram shown in Figure 1, was designed employing two photodiode, expecting that those arrangement afforded an improvement in sensitivity. Once the appropriate operational condition was established, assays were implemented to verify if a gain in signal magnitude occurred or not. The assay was done coating the sensitive surface of the photodiode (D_1, Figure 1), using a piece of black cloth. After recording the generated signal, the coating piece was removed and the assay was repeated, yielding the results shown in Figure 6.

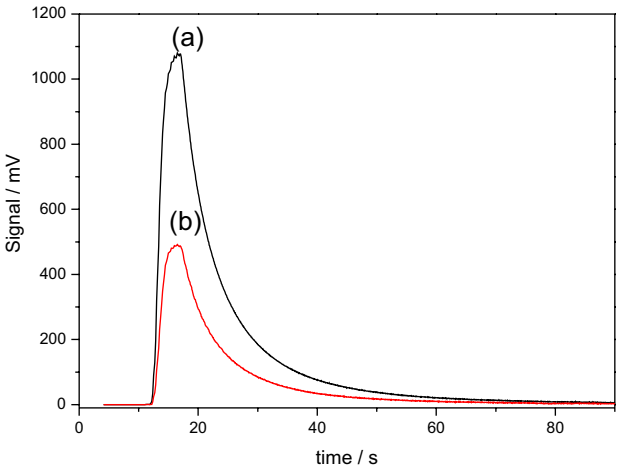

Figure 6. Effect related to the use of two photodiodes. Records (a) and (b) correspond to the measurements carried out using two and only one photodiode, respectively.

Ideally, the maximum magnitude of the record (a) would be the double of the record (b), nevertheless the optical coupling of the two photodiodes to the luminescence reactor coil is not identical, so the answers are different, but there is a significant gain in signal using the summing strategy.

Potential interferences

Usually, folic acid pill includes as excipients lactose, starch, stearate and ascorbic acid, so a set of assays was carried out in order to evaluate if these compounds cause or not interference. The tests were performed using a 0.5 mg mL^{-1} folic acid standard solution with and without the potential interferences. A variation of ± 5% in analytical signal has been used as a criterion to define if a concomitant compound cause or not interference.[25] Taking as reference, the signal related with the folic acid standard solution without interferings, we observed that excepting ascorbic acid, the tolerance was higher than 1000 times the folic acid concentration used for tests. Additional assays shown that for ascorbic acid, the tolerance was 10 times the concentration of folic acid used in this assay (0.5 mg mL^{-1}), which is acceptable for this pharmaceutical type.

Sample analyses and performance comparison

Intending to evaluate the practicality of the proposed analytical system, a set of folic samples were analyzed. The records of the signals readout generated by the luminometer (Figure 1) are shown in Figure 7, where we can see that the signals have high reproducibility, indicating an adequate overall performance.

Taking as the measurement parameter, the maximum values of the records related with the folic acid standard solutions, we derived the following linear equation: Signal (mV) = (–310.54 ± 3.92)[FA] + (1226.20 ± 0.82) (R^2 = 0.993). This equation is related to the folic acid concentration ranging from 0.1 to 1.0 mg mL^{-1}, nevertheless extending the concentration range up to 2.0 mg mL^{-1}, we observed a decrease of 20% in the slope of the analytical

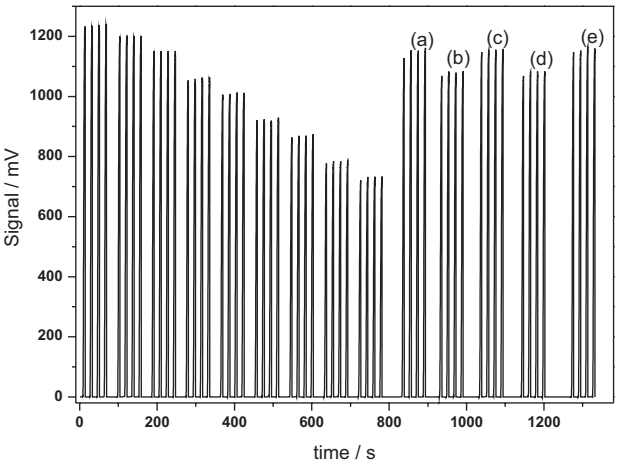

Figure 7. Records of the luminometer readout. From left folic acid standard solution 0.0, 0.1, 0.2, 0.5, 0.7, 1.0, 1.3, 1.7 and 2.0 mg mL^{-1}; followed of five folic acid samples.

curve and a loss of linearity (R^2 = 0.985). Analyzing the Figure 7, we observe that to carry out 52 determination, the time interval elapsed was 1200 s, therefore we can deduce that the proposed system would be able to carry out 156 determination *per* hour.

In Table 3 is shown a summary of the main parameters, usually employed to evaluate the performance of the analytical procedures based on flow analysis process. Analyzing these data, we observe that the sensitivities of the procedures represented by the reference numbers 5 and 8, are higher than that of the proposed procedure. This would be expected, owing to the referred procedures employed equipments furnished with photomultiplier, which is a sophisticated and very sensitive device, while in the current work was employed a simple homemade luminometer.

The working range of the proposed procedure is enough for the determination of folic acid in

Table 3. Performance comparison

Parameter	Proposed procedure	Reference 5	Reference 6	Reference8	Reference26
Linear range	0.1-1.0[a]	0.01-15[b]	0.1-21[b]	0.4-353.0[c]	0.1-40.0[b]
Linearity (r)	0.996	0.999	–	0.999	0.997
Limit of detection	0.046[a]	3.5[c]	0.03[b]	0.26[c]	–
Detection	photodiode	PMT[d]	PMT[d]	PMT[d]	fluorimetry
RSD[e] / %	< 0.5	< 2.5	< 4	2.6	3.0
Consumption[f] / mg	$K_3[Fe(CN)_6]$ 66 µg luminol 64 µg	–	$K_3[Fe(CN)_6]$ 7.8 µg luminol 708 µg	Co 600µg SO_5^- 1300 µg	-
Sampling rate[g]	156	30	30	30	25
Waste[h] / mL	< 0.9	16.0	16.0	10.0	1.4

[a]Concentration in mg mL^{-1}; [b]concentration in µg mL^{-1}; [c]ng mL^{-1}; [d]photomultiplier; [e]relative standard deviation; [f]reagent consumption *per* determination; [g]number of determination *per* hour; [h]waste generation *per* determination.

Table 4. Results of sample analysis

Sample	Labeled / (mg pill^{-1})	Proposed / (mg pill^{-1})[a]	Diluted sample / (mg mL^{-1})	Added value / (mg mL^{-1})	Found / (mg mL^{-1})	Recovery / %
A	5	6.21 ± 1.02	0.193	0.30	0.452	86.33
B	10	11.88 ± 0.48	0.495	0.30	0.825	110.00
C	5	5.85 ± 0.25	0.199	0.30	0.522	107.67
D	10	11.84 ± 0.45	0.487	0.30	0.802	105.00
E	5	5.65 ± 0.57	0.172	0.30	0.481	103.00

[a]Concentration *per* pill, determined employing the proposed procedure. Results are average of four consecutive measurements.

pharmaceutical formulation without any sample treatment prior to analysis. Reagent consumption is better than that of reference 8, while the relative standard deviation of the proposed procedure is better than those of the papers related in this Table 3. The sampling rate and waste generation are highly favorable to the current work. Comparing with the procedure based on fluorimetry,[26] we observe that the volumes of waste generate are similar, but the others parameters compare favorably to the current work.

Intending to prove the feasibility of the proposed procedures, samples of pharmaceutical formulations of folic acid were analyzed. To allow accuracy assessment, the samples were also processed using the standard addition methodology and results are summarized in Table 4. Analyzing these data, we observe that a recovery within the range of 86 to 110% was achieved, which is considered acceptable.

Conclusions

An overview on the results presented in the precedent sections, allow us to conclude that the overall performance of the analytical system is excellent. The low volume of waste generated would be considered as confirmation that environmentally friendly condition, according to the green analytical chemistry guidelines was fulfilled.[18,19]

The high analytical throughput, achieved using a cost-effective equipment, which exploiting the facilities due to the multicommuted flow analysis approach, enabled saving reagent without sacrificing quality of results. This allow us to concluded that the proposal could became an effective alternative to the existing analytical procedures for folic acid determination in pharmaceuticals.

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