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ORIGINAL PAPER

Development of Flow Injection Spectrofluorimetric Detection System for the Determination of Homocysteine

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Abstract In this work, a new simple and sensitive flow injection method is developed for the determination of homocysteine with spectrofluorimetric detection technique. This method is based on the oxidation of homocysteine with Tl (III) in acidic media, producing fluorescence reagent, $TlCl_3^{2-}$ (λ_{ex}=237 nm, λ_{em}=419 nm). The effects of chemical parameters (including pH of the solutions, the buffer, Tl (III) and potassium chloride concentrations), instrumental parameters (such as flow rate of the solutions, reaction coil length, and sample loop volume) and temperature on the fluorescence intensity as an analytical signal are studied and optimized. In the optimum conditions of the above variables, homocysteine can be determined in the range 4.0×10^{-7}–40.0×10^{-6} M with the LDR from 4.0×10^{-7} to 25.0×10^{-6} M. The detection limit (with S/N=3) is 6.0×10^{-8} M of homocysteine and precision for the injection of 5.0, 10.0 and 15.0 μM of homocysteine are 0.8%, 1.5% and 2.5% (n=10) respectively. The rate of analysis is 90 samples per hour. The influence of potential interfering substances, including amino acids and carbohydrates is also studied. The proposed method has been successfully used for the determination of homocysteine in the real sample (blood serum and tap water) matrix.

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Introduction

Homocysteine (2-amino-4-mercaptobutyric acid; Hcys), a sulfur-containing amino acid is an intermediate compound formed during the metabolism of methionine to cysteine. Although it is not found directly in the diet, it can be biosynthesized by demethylation of the essential amino acid methionine [1]. Homocysteine has several important roles in physiological matrices. Genetic or nutritional deficiencies in Hcys metabolism lead to hyperhomocysteinemia and are associated with cardiovascular and neurodegenerative disorders such as dementia and Alzheimer's disease [2, 3].

It has also been discovered that hyperhomocysteinemia, whereby too much homocysteine is present in the body, has been associated with both folate and cobalamine deficiencies, and also with early pregnancy loss, neural tube defects, mental disorders, cognitive impairment in the elderly, psoriasis, dystonia [4, 5], pregnancy complications and some tumors [6]. The usual concentration ranges for hyperhomocysteinemia are split into three categories; 15–30 μM of total homocysteine is associated with moderate hyperhomocysteinemia, 30–100 μM is intermediate and up to or over 100 μM means the patient has severe hyperhomocysteinemia [7]. Hence, the determination of homocysteine as an important biomarker for a wide range of diseases has gained high interest in the biomedical community over recent years [1–7].

Chromatographic methods are commonly used for the determination of homocysteine in biological samples. Gas

chromatography has been very scarcely used because of the high polarity of Hcys and related compounds, which makes necessary a previous derivatization to increase volatility [8]. Liquid chromatography with UV [9, 10] or fluorescence [11, 12] detection has been frequently used, although derivatization treatments in order to introduce chromophore or fluorofore groups are required. LC–MS provides a useful analytical tool for the identification and quantification of these compounds [13].

In this paper, a new flow injection spectrofluorimetric method is proposed for the determination of homocysteine based on the oxidation of homocysteine with Tl (III) in acidic media, producing fluorescence reagent, $TlCl_3^{2-}$. The reaction was monitored by measuring the fluorescence intensity of the $TlCl_3^{2-}$ (λ_{ex}=237 nm, λ_{em}=419 nm). The proposed method was successfully used to determine of homocysteine in the real samples (blood serum and tap water).

Experimental

Apparatus and Reagents

A diagram of the flow system employed is shown in Fig. 1. The fluorescence of the $TlCl_3^{2-}$ (λ_{ex}=237 nm, λ_{em}=419 nm) was measured with a Varian model Cary-Eclipse spectroflourimeter equipped with a flow through cell (40 μL inner volumes). A 4-channel peristaltic pump (Gilson, Minipuls 3) with two silicon rubber tubes (1.02 mm i.d.) was used. PTFE mixing joints and PTFE tubing (0.5 mm i.d.) were used for the connections and the reaction coil. The controlled water bath (home made) was used for controlling the temperature. Metrohm model 691 pH-meter was used for the determination and adjusting the pH of the solutions. Sample solutions were injected using a six position rotary Rheoyne valve with a sample loop of 204 μL.

All the solution was prepared using reagent grade chemicals and distilled water was used throughout.

Tl(III) standard solution (0.01 M) was prepared by dissolving 0.4444 g of $Tl(NO_3).3H_2O$ (Merck) in distilled water and diluting to 100 mL in volumetric flask. Working standard solution was freshly prepared by diluting the stock solution with water. Buffer solutions was prepared by using universal buffer (Britton-Robinson) and phosphate buffer systems. Potassium chloride solution (1.0 M) was prepared by dissolving 7.45 g of KCl (Merck) in water and diluting to 100 mL in a volumetric flask. Homocysteine solution (2.00×10^{-3} M) was prepared by dissolving 6.70×10^{-3} g of DL-Homocysteine (Fluka) in degassed distilled water and diluting to 25 mL in volumetric flask.

Recommended Procedure

As shown in Fig. 1 each solution containing carrier (KCl in buffer solution, R_1) and a mixture of the Tl(III) plus phosphate buffer and potassium chloride (R_2) previously thermostated at an appropriate temperature (20 °C) is pumped at 0.90 $mLmin^{-1}$ via a peristaltic pump. The standard solution containing 0.40×10^{-6}–40.0×10^{-6} M of Hcyc was injected into a carrier stream via sample injection valve. The sample solution was directly treated with a mixture of the Tl(III) plus phosphate buffer and potassium chloride, and then passed to the sample flow cell of the spectrofluorimeter via reaction coil, where the fluorescence of the $TlCl_3^{2-}$ (λ_{ex}=237 nm, λ_{em}=419 nm) was measured as Hcys concentration. The concentration of Hcys was evaluated from the peak height measurements by using a calibration curve prepared from the results obtained on standards.

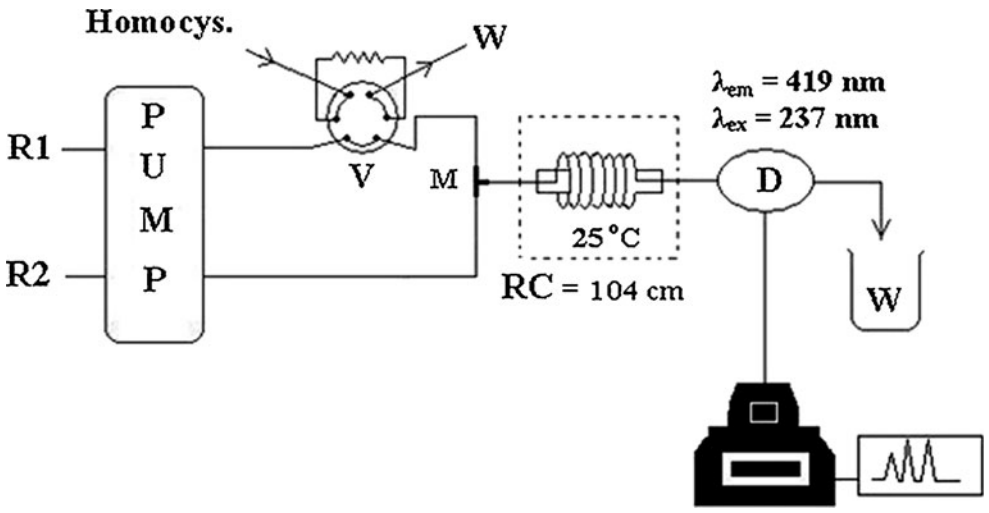

Fig. 1 Schematic representation of the flow injection manifold employed for the determination of Homocysteine. R_1: carrier, R_2: a mixture solution of the Tl^{3+}, Buffer and potassium chloride, V: injection valve, M: mixing zone, RC: reaction coil, D: detector, W: waste

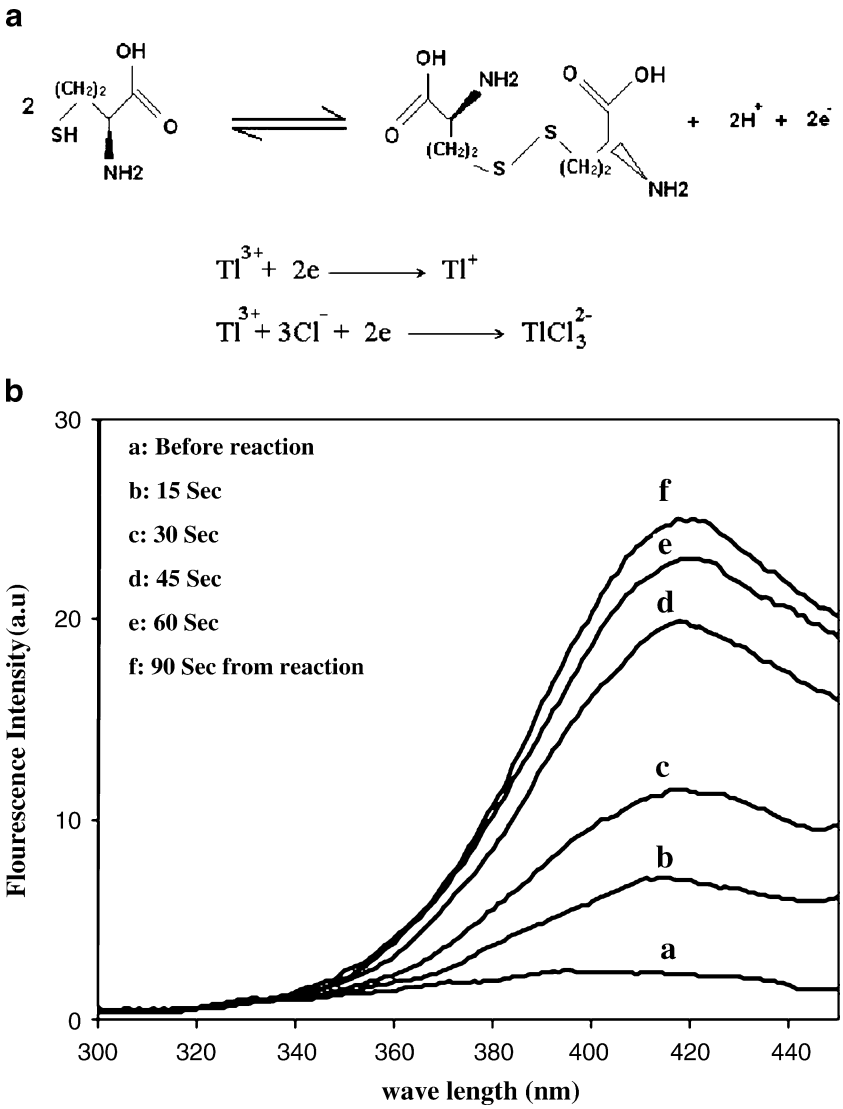

Fig. 2 **a** The redox reaction of Homocysteine and Tl(III), **b** Fluorescence spectrum of the redox reaction at different time intervals. Conditions: Tl (III), 2.0×10^{-5} M; pH, 6; KCl, 0.15 M; Homocysteine, 2.0×10^{-5} M

Real Sample Analysis

The new blood serum specimen was centrifuged at 4500 cycles per min for 15 min. Three samples (1.0 mL volume for each) were treated with HCl for deproteination and then were centrifuged for 15 min at the rate of 13000 cycles per min [14]. This solution was applied to the reaction with reagents. Tap water sample was used without any pretreatment.

Results and Discussion

Optimization of the Variables

The results of our studies showed that the Tl(III) can be reduced in acidic media by homocysteine. The product of this redox reaction is fluorescence reagent, $TlCl_3^{2-}$ (Fig. 2a) that can be fluoresce at λ_{em}=419 nm when excited at λ_{ex}=237 nm (Fig.2b). Therefore, the fluorescence intensity at 419 nm was

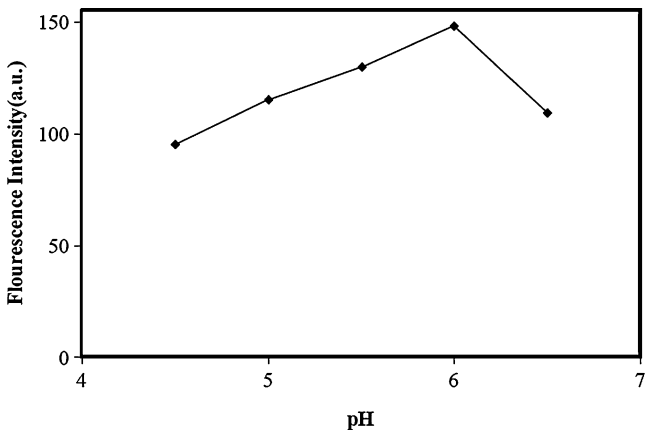

Fig. 3 pH effect on the fluorescence intensity. Conditions: Tl (III), 2.00×10^{-5} M; KCl, 0.15 M; Homocysteine, 2.00×10^{-5} M; flow rate, 0.20 mLmin^{-1}; reaction coil length, 104 cm; injection volume, 100 μL and t, 25 °C

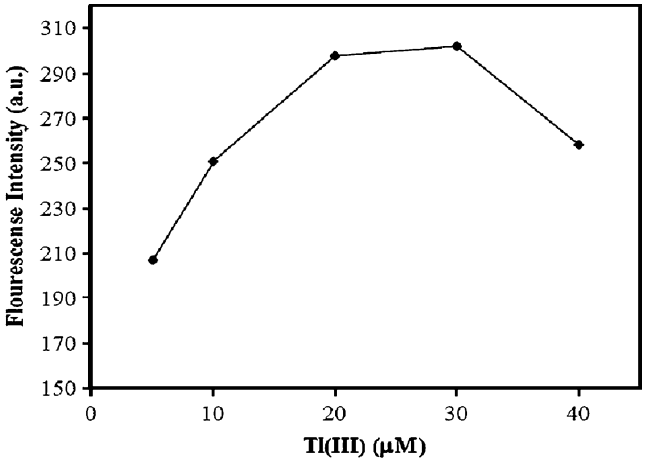

Fig. 4 Effect of Tl (III) concentration on the fluorescence intensity. Conditions: pH, 6.0, (buffer concentration, 0.30 M Phosphate ion); KCl, 0.15 M; Homocysteine, 2.00×10^{-5} M; flow rate, 0.20 mLmin^{-1}; reaction coil length, 104 cm; injection volume, 100 μL and t, 25 °C

used as analytical signal. Preliminary tests were carried out with the aid of different flow assemblies to select optimal manifold configuration. The assembly in Fig. 1 was selected as the best configuration. In order to optimize the flow injection system, the influence of the reagents concentration and temperature as well as manifold variables on the sensitivity were studied.

Considering experimental results, the reaction affected by the pH of the medium and could run well in the solution when they buffered in the special pH. Therefore, the first study was the influence of the pH of the buffer solution on the fluorescence intensity. As Fig. 3 shows fluorescence intensity of the reaction goes up till pH 6 and then goes down. For getting good sensitivity and low detection limit, pH 6 was selected as optimum pH of the buffer solution for next study.

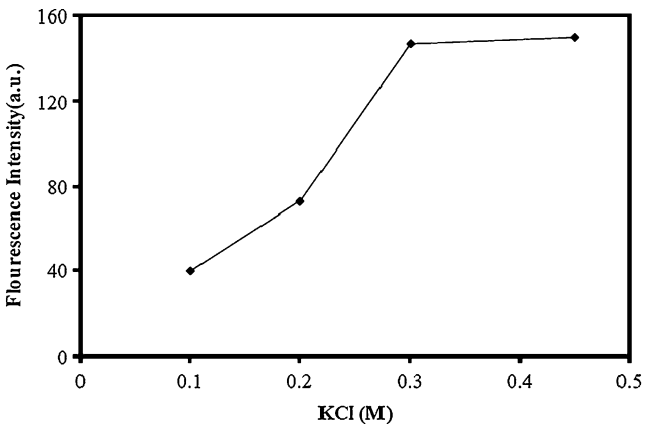

Fig. 5 Influence of potassium chloride concentration on the sensitivity. Conditions: pH, 6.0, (buffer concentration, 0.30 M Phosphate ion); Tl (III), 2.00×10^{-5} M; Homocysteine, 2.00×10^{-5} M; flow rate, 0.20 mLmin^{-1}; reaction coil length, 104 cm; injection volume, 100 μL and t, 25 °C

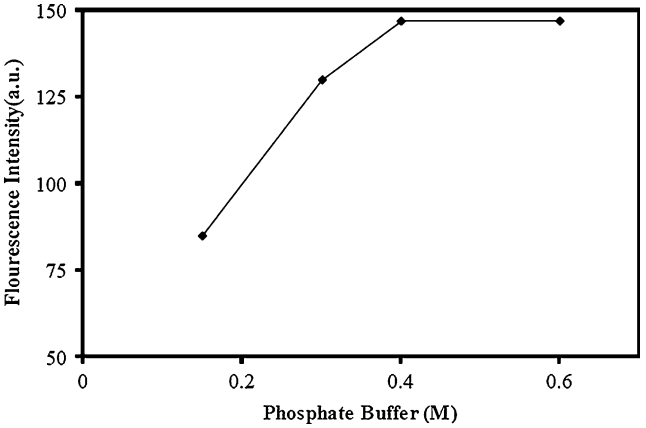

Fig. 6 Influence of buffer concentration on the sensitivity. Conditions: pH, 6.0; Tl (III), 2.00×10^{-5} M; KCl, 0.30 M; Homocysteine, 2.00×10^{-5} M; flow rate, 0.20 mLmin^{-1}; reaction coil length, 104 cm, injection volume; 100 μL and t, 25 °C

Figure 4 shows the influence of Tl(III) concentration in the range of 5.0×10^{-6} to 4.0×10^{-5} M, (conditions were shown in the legend of the figures). The results show that increasing the Tl(III) concentration leads to increase the sensitivity up to 2.0×10^{-5} and then was decreased at higher concentration. Therefore, 2.0×10^{-5} M of Tl(III) was selected as optimum concentration.

The influence of chloride ion concentration on the peak height was studied for the potassium chloride concentration range from 0.10 to 0.45 M, with 2.0×10^{-5} M Tl(III) and 2.0×10^{-5} M Hcys (Fig. 5). The results show that increasing the KCl concentration up to 0.30 M leads to increasing the sensitivity whereas, higher concentration does not affect the sensitivity. Therefore, 0.30 M KCl concentrations were selected for further study.

Figure 6 shows the influence of phosphate buffer concentration on the sensitivity in the range of 0.15 to 0.60 M, with 2.0×10^{-5} M Tl(III), 0.30 M KCl and pH 6 at

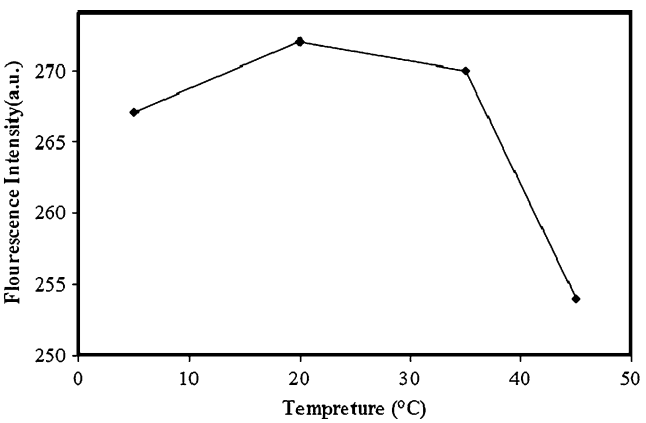

Fig. 7 Effect of the temperature on the sensitivity. Conditions: pH, 6.0; buffer phosphate concentration, 0.40 M; Tl (III), 2.00×10^{-5} M; KCl, 0.30 M; Homocysteine, 2.00×10^{-5} M; flow rate, 0.20 mLmin^{-1}; reaction coil length, 104 cm; injection volume, 100 μL

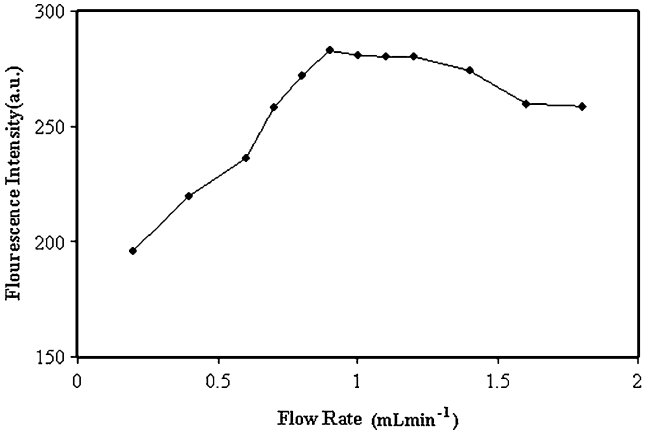

Fig. 8 Effect of pump flow rate on the sensitivity. Conditions: pH, 6.0; buffer phosphate concentration, 0.40 M; Tl (III), 2.00×10^{-5} M; KCl, 0.30 M; Homocysteine, 2.00×10^{-5} M; reaction coil length, 104 cm; injection volume 100 μL and t, 20 °C

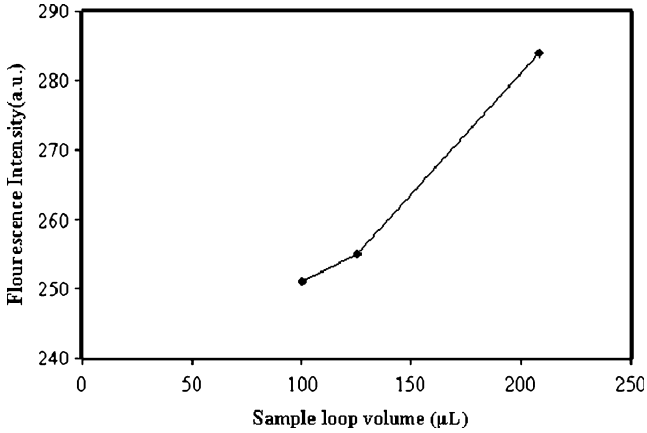

Fig. 10 Effect of the injection volume on the sensitivity. Conditions: pH, 6.0; buffer phosphate concentration, 0.40 M; Tl (III), 2.00×10^{-5} M; KCl, 0.30 M; Homocysteine, 2.00×10^{-5} M; flow rate, 0.90 mLmin^{-1}; reaction coil length, 104 cm; t, 20 °C

25 °C. According to the results, the peak height goes up as buffer concentration increases up to 0.40 M, and then level up. Therefore, 0.40 M phosphate buffer concentration was selected.

The effect of the temperature on the peak height was studied in the range of 5.0 to 45.0 °C, with 2.0×10^{-5} M Tl(III), 0.30 M KCl, 0.40 M phosphate buffer, pH 6 and 2.0×10^{-5} M Hcys (Fig. 7). Based on the results, increasing the temperature up to 20.0 °C cause increase in the sensitivity and then was decreased at higher temperature due to decomposition of the reagents. Therefore room temperature (20.0 °C) was used for further study.

The influence of manifold variables on the sensitivity (pump flow rate, length of the reaction coil and sample loop volume) was studied with the optimized reagents concentration and 2.0×10^{-5} M Hcys. The peak height depends on the residence time of the sample zone in the system; e.g. on the total flow rate and the length of the reaction coil. The effect of the flow rate was checked over the range 0.20–1.70 mLmin^{-1}. The results show that the peak height grows as flow rate raises up to 0.90 mLmin^{-1}, and then decrease (Fig. 8). This is due to the fact that at higher flow rate the residence time of mixture of the reagents is reduced and thus the rate of the $TlCl_3^{2-}$ production decreased, causing a drop in the peak height. Also at very low flow rates, peaks of the analytical signal are broad and leading to decrease in the peak height. From the result, pump flow rate of 0.90 mLmin^{-1} was chosen for study.

The influence of the length of the reaction coil on the sensitivity was investigated with flow rate of 0.90 mLmin^{-1}. Considering the results, increasing the length of the reaction coil from 50 to 160 cm, initially it caused increasing the

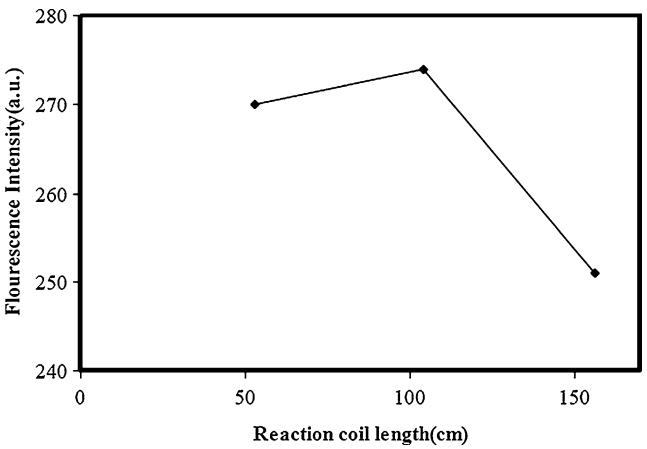

Fig. 9 Effect of the length of the reaction coil on the sensitivity. Conditions: pH, 6.0; buffer phosphate concentration, 0.40 M; Tl (III), 2.00×10^{-5} M; KCl, 0.30 M; Homocysteine, 2.00×10^{-5} M; flow rate, 0.90 mLmin^{-1}; injection volume, 100 μL and t, 20 °C

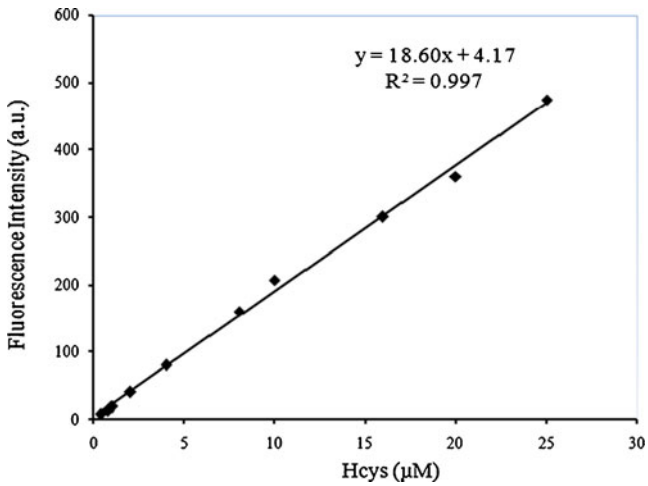

Fig. 11 Calibration curve for the determination of Homocysteine in the optimum conditions. Concentration range: 0.40 to 25.0 μM of Homocysteine. Conditions: pH, 6.0; buffer phosphate concentration, 0.40 M; Tl (III), 2.00×10^{-5} M; KCl, 0.30 M; flow rate, 0.90 mLmin^{-1}; reaction coil length, 104 cm; injection volume, 208 μL and t, 20 °C

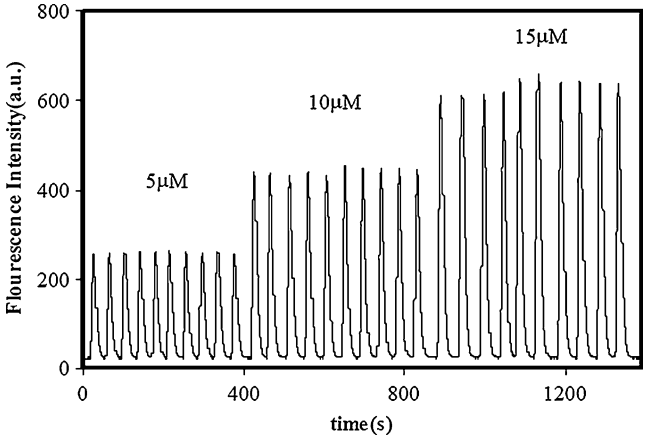

Fig. 12 Precision and reproducibility of the obtained analytical signals. (Ten replicate injections of 5.0, 10.0 and 15.0 μM of Homocysteine in the optimum conditions). Conditions: pH, 6.0; buffer phosphate concentration, 0.40 M; Tl (III), 2.00×10^{-5} M; KCl, 0.30 M; flow rate, 0.90 $mLmin^{-1}$; reaction coil length, 104 cm; injection volume, 208 μL and t, 20 °C

analytical signal (up to 104 cm) and then it decreased (for >104 cm) (Fig. 9). By increasing the length of the reaction coil, the reaction between the reagents goes more ahead, but because of the broadening, the height of the peaks as analytical signals reduces. Therefore, a 104 cm was chosen as the optimum length of the reaction coil.

The sample volume injected into the carrier line has a significant effect on the peak height (Fig. 10). The signal rises with increasing sample volume up to 200 μL. In addition, using sample volume larger than 208 μL leading to peak broadening and tailing. Therefore, a sample volume of 208 μL was selected for further experiments because of sharper peaks.

Analytical Parameters

Under the optimized conditions and at 20.0 °C the peak height obtained was proportional to the Hcys concentration in the range of 0.40×10^{-6}–40.0×10^{-6} M. The linear dynamic range is in this range of 0.40×10^{-6}–25.0×10^{-6} M of Hcys (Fig. 11). The detection limit for the determination of Hcys was 0.06×10^{-6} M (S/N=3) and relative standard deviations (n=10) for 5.0×10^{-6}, 10.0×10^{-6} and 15.0×10^{-6} M of Hcys were 0.8, 1.5 and 2.5% respectively (Fig. 12). The rate of analysis (number of samples injected in 1 h) in the optimum conditions was 90 samples per hour.

Table 1 The effect of foreign substances on the determination of Homocysteine

Species	Tolerance limit ($W_{Species}/W_{Hcys}$)
Glycine, Alanine, L- Lysine, L-Leucine, L-Proline, L-Threonine, L-Valine, Cystine, L-Asparagine, L-Serine, Tartaric acid, Citric acid, NaCl, EDTA, Sucrose, Glucose, Fructose, Lactose, Zn^{2+}, Mg^{2+}, Ca^{2+}, SO_3^{2-}, F^-, $Cr_2O_7^{2-}$, NH_4^+, SCN^-, IO_4^-, ClO_3^-, BrO_3^-, Ce^{4+}, CO_3^{2-}	1000[a]
L-Asparatic acid, Urea, Cd^{2+}, HCO_3^-	200
L-Hystidine, Methionine,	10

[a] Maximum concentration of the reagent tested

Table 2 Data for the determination of Homocysteine in Tap water

Sample	Homocysteine (added), μM	Homocysteine (detected, n=3), μM	%R
1	1.00	1.04(±0.04)	104.0
2	2.00	2.08(±0.13)	104.0
3	4.00	3.66(±0.12)	91.5
4	6.00	5.83(±0.32)	97.5
5	8.00	8.19(±0.20)	102.0

Interference Study

Under the optimized conditions, the influence of several species (amino acids, cations and anions) on the determination of 2.0×10^{-5} M Hcys was studied. The tolerance limit was defined as the interference ions cause less than ±3% relative error for the Hcys determination. The results are summarized in Table 1.

Applications

The present method was successfully applied to the determination of Hcys in tap water and blood serum samples. In view of the unknown composition of the samples, equivalent portions of each sample were analyzed for Hcys contents by standard addition method. All samples were injected three times and their standard deviations and recoveries were calculated which are shown in the tow last columns of Tables 2 and 3. The results show good recovery and also good reproducibility in the spiked samples of unknown real sample matrix.

Table 3 Data for the determination of homocysteine in blood serum

Sample	Homocysteine (added), μM	Homocysteine (detected, n=3), μM	%R
1	–	5.75(±0.40)	–
2	1.00	6.73(±0.09)	98.0
3	2.00	7.60(±0.40)	92.5
4	4.00	9.45(±0.50)	93.0
5	6.00	11.60(±0.15)	97.5

Conclusion

The new method described is significant with respect to the development of a simple FIA method for the determination of trace amounts of Hcys in the real samples. Its simplicity and reproducibility are coupled with the high speed and safety analysis of the FIA technique.

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