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SHORT COMMUNICATION

FLUORIMETRIC DETERMINATION OF PHENOTHIAZINE DERIVATIVES BY PHOTOOXIDATION IN A FLOW-INJECTION SYSTEM

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Summary—Flow-injection analysis (FIA) was combined with photochemically induced fluorescence (PF) detection for the determination of four phenothiazine derivatives, including unsubstituted phenothiazine, thionine, Azure A and Methylene Blue. The working analytical parameters (flow-rate, injected volume, photoreactor length) were optimized. Linear calibration graphs were obtained over about two orders of magnitude, with relative standard deviation within the range 1–2.3%. Limits of detection were between 13 and 35 ng/ml, according to the compound. The FIA-PF method was applied to the determination of phenothiazines in urine samples. Mean recoveries ranged from 94 to 117%.

INTRODUCTION

Phenothiazine derivatives are of increasing interest in medicine. They have been used primarily as psychotropic drugs.[1,2] Some members of this group possess a variety of other biomedically important properties, including antiemetic, antipruritic, anti-nausea, analgesic and antitumoral activity.[3–5] Phenothiazine dyes such as Methylene Blue, Azure A, Methylene Violet, Thionine, Methylene Green and Nile Blue A have been studied for their photo-chemotherapeutic effects against carcinomas. A number of analytical methods have been developed for determining phenothiazine derivatives.[6–41] Thin-layer chromatography (TLC),[6–12] gas chromatography (GC),[13–15] high-performance liquid chromatography (HPLC),[16–24] flow-injection analysis (FIA),[25–28] colorimetry,[29] ultraviolet (UV)–visible spectrophotometry[30–35] and spectrofluorimetry[36–41] have been reported. Several of these methods present drawbacks, such as lack of sensitivity or the use of chemical oxidation or complexation reactions, which are time-consuming processes.

In recent years, several researchers have utilized the photochemical reactivity of phenothiazine derivatives to improve the sensitivity and rapidity of fluorescence detection.[22–28,40,41] Using optimal conditions, phenothiazines can be rapidly photoxidized into strongly fluorescent photoproducts. For instance, Scholten *et al.*[22,23] employed photochemical-fluorescence detection in HPLC for quantifying mezoridazine, sulforidazine, and thioridazine at the pg level. Brinkman *et al.*[24] applied the same method to the determination of fenergan, largactil, and related phenothiazines. Chen *et al.*[25] determined chlorpromazine, promethazine and perphenazine by irradiating either a flow cell in a stopped-flow injection system or a reaction coil in a FIA system with UV light. The same authors described the simultaneous analysis of chlorpromazine and promethazine at the μg/ml level, using the same FIA system with photochemical-fluorimetric detection.[26] Martinez Calatayud and Gomez Benito,[27] and Mellado Romero *et al.*[28] also applied photochemical derivatization to the spectrofluorimetric determination of chlorpromazine and promethazine by FIA. We recently developed a simple photochemically induced fluorescence (PF) method for the analysis of five selected phenothiazines in aqueous bulk solutions.[41] We investigated the influence of pH and UV irradiation time on the performances of the technique.

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In the present work we coupled FIA with PF detection for determining four phenothiazines including unsubstituted phenothiazine, Azure A, Methylene Blue and thionine (structures in Fig. 1). We applied the FIA-PF method to the analysis of these phenothiazines in human urine samples.

EXPERIMENTAL

Reagents

Phenothiazine (PHE) (**1**), thionine (THI) (**2**), azure A (AZU) (**3**) and Methylene Blue (MB) (**4**) (Fig. 1) were purchased from Aldrich (Milwaukee, MI, U.S.A.) and from Fluka (Buchs, Switzerland). The buffer solutions (pH 1–13) were obtained from Merck. Solvents used were distilled and deionized water and ethanol (Merck, analytical-reagent grade).

Apparatus

Fluorescence measurements were performed on a Kontron SFM25 spectrofluorimeter, equiped with a HPLC fluorescence cell accessory, and interfaced with a GEOCOM microcomputer model CPC 1420E. Data acquisition and data analysis were performed by using a SFM data acquisition program. The FIA-PF system was described previously.[42] The experimental set-up included an Ismatec IPN-4 peristaltic pump, a rotary injection valve and a 254 nm low-pressure mercury lamp irradiating a PTFE reactor (0.5 mm i.d.).

The injected samples were propelled with an aqueous carrier and were irradiated in the PTFE photoreactor before fluorimetric detection. The effect of PTFE photoreactor length, flow-rate, residence time and injected volume on the fluorescence signals were examined for several concentration of phenothiazines in order to provide maximum responses and minimum band broadening.

Procedure

Stock solutions of phenothiazines ($10^{-3}M$) were prepared by dissolving the corresponding compounds in water or ethanol. Working solutions ($10^{-5}M$) were obtained by serial dilution, using various volumes of buffer solutions for obtaining the convenient pH values.

The carrier used was water in all instances. Photochemically induced fluorescence measurements of phenothiazines were performed with the UV lamp on. For unsubstituted phenothiazine, all working solutions contained 1% of ethanol, for solubility reasons. In all cases, the solutions were buffered at a pH value depending of the compound. The effect of pH on the PF signals of phenothiazines was investigated previously.[41] Triplicate measurements were made to evaluate the reproductibility for each analyte. Fluorescence measurements were performed at constant excitation and emission wavelengths corresponding to the phenothiazine photoproducts. The sensitivity of the spectrofluorimeter was maintained at 10, and a 500–700 V voltage range was utilized.

Analytical figures of merit were obtained in the range 0.05–4.0 μg/ml for the phenothiazine working solutions. Recoveries from urine sample solutions were evaluated by using the standard addition procedure, after diluting urine samples 125–500 times in water.

RESULTS AND DISCUSSION

Effects of variables

The effects of several variables on PF intensity and width of the flow-injection peaks of phenothiazines were investigated. It was found that the injected volume, the flow-rate and the reactor length produced significant changes in PF signals. We have presented in Table 1 the range of investigation of these parameters and

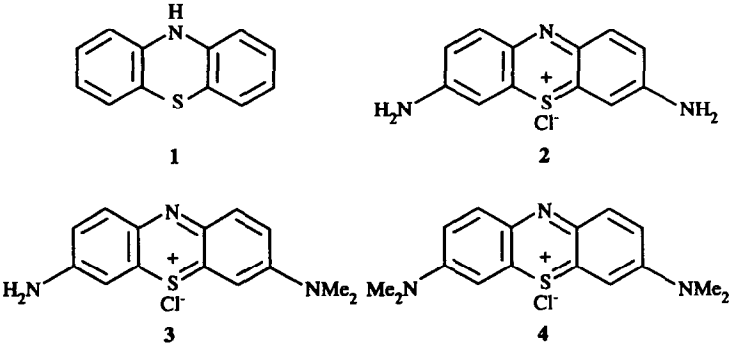

Fig. 1. Structures of phenothiazine derivatives under study.

Table 1. Optimum analytical parameters for the flow-injection photochemically induced fluorescence of phenothiazines

Analytical parameter	Studied range	Selected value PHE	THI	AZU	MB
λ_{ex} (nm)*	—	310	360	345	345
λ_{em} (nm)*	—	382	523	485	485
pH	1–13	2.0	10.0	13.0	13.0
Flow rate (ml/min)	0.6–4.0	4.0	2.7	3.0	2.0
Residence time (sec)	0.1–42.7	5.9	26.2	15.7	35.3
Volume injected (μl)	47–300	300	300	300	200
Reactor length (cm)	50–150	50	150	100	150
Sensitivity†	1–10	10	10	10	10
Voltage (V)†	500–700	550	650	600	700

*Analytical excitation and emission wavelengths.
†Instrumental characteristics.

the optimum analytical values corresponding to maximum PF response and minimum peak broadening. Figure 2 shows that the photochemically induced fluorescence intensity increased significantly for injected volumes larger than 100 μl. Changes in flow-rate also produced a variation of PF intensity. The curves in Fig. 3 indicate a decrease in PF intensity when the flow-rate was increased, because of shorter UV irradiation times. We used a short reactor length (L_r) for neutral phenothiazine (L_r = 50 cm), but longer ones for the charged phenothiazine derivatives (L_r = 150 cm for THI and MB, and 100 cm for AZU).

This can be related to the difference in the optimum irradiation times observed in stationary solution for phenothiazine itself (5 sec), and for charged derivatives (4–17 min).[41] Residence times of phenothiazines were relatively short ranging from 5.9 to 35.5 sec (Table 1).

It can be seen that our FIA-PF approach is improved relatively to previous FIA methods used for the determination of other phenothiazines,[25–28] since an optimum geometry has been developed for the photochemical reactor, and shorter residence times have been applied in the present work.

Analytical data

Table 2 summarizes the analytical data for the FIA-PF determination of phenothiazines. These figures were obtained in the range 0.01–4.0 μg/ml, from at least three or four measurements. Log–log plots of the fluorescence intensity of phenothiazine photoproducts *vs.* initial concentration were established over a concentration range of at least two orders of magnitude. The correlation coefficient values range between 0.993 and 0.999, showing the excellent precision of linear calibration curves. The relative standard deviation values are between 1.0 and 2.3%. The limits of detection (LOD) are low, ranging from 13 ng/ml for phenothiazine to 35 ng/ml for Methylene Blue.

The results obtained by our FIA/PF method present several analytical advantages against

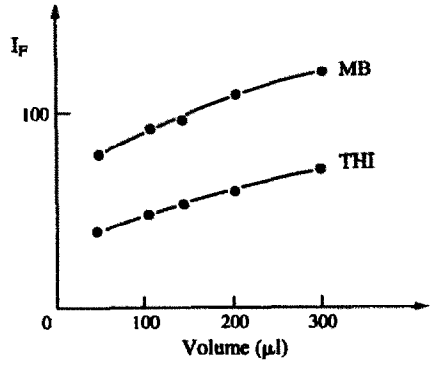

Fig. 2. Effect of sample injection volume on the photochemically-induced fluorescence signal of Methylene Blue ([MB] = 3.19 μg/ml, flow rate = 2.0 ml/min) and thionine ([THI] = 2.8 μg/ml, flow rate = 2.7 ml/min); reactor length = 150 cm.

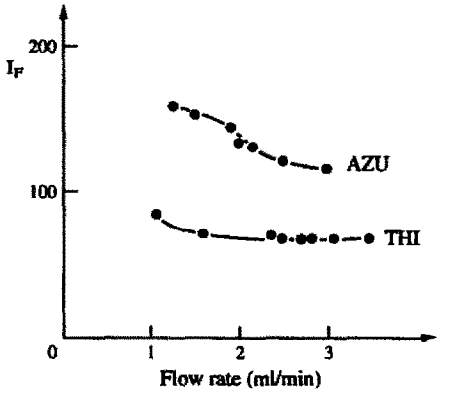

Fig. 3. Effect of flow-rate on the photochemically induced fluorescence signal of 2.9 μg/ml Azure A (reactor length = 100 cm) and 2.8 μg/ml thionine (reactor length = 150 cm); injected sample volume 300 μl.

Table 2. Analytical data for the flow-injection photochemically induced fluorescence determination of phenothiazines

Compound	Concentration range (μg/ml)	Regression equation* A	B	Correlation coefficient	LOD† (ng/ml)	RSD‡ (%)
Phenothiazine	0.01–2.0	1.58	0.89	0.996	13	1.0
Thionine	0.05–2.0	1.39	1.04	0.998	35	2.3
Methylene Blue	0.06–2.0	1.37	1.10	0.999	16	2.2
Azure A	0.05–4.0	2.08	1.01	0.993	20	1.7

*$\log I_f = A + B\log c$; I_f = relative fluorescence signal; c = analyte concentration (μg/ml).

†LOD = Limit of detection, defined as the concentration of analyte giving a signal-to-noise ratio of 3.

‡RSD = Mid-range relative standard deviation ($N = 6$).

the previous ones described in the literature. First, our LOD values compare rather favourably with those reported in the literature (LOD = 5–25 μg/ml, determined for various phenothiazines by TLC-SIMS,[15] 50 ng/ml for promethazine and 20–31 ng/ml for chlorpromazine, determined by FIA.[25,27,28] Secondly, our reactor was completely exposed to UV irradiation, with lengths of PTFE tube coiled close to the lamp ranging from 50 to 150 cm, whereas in the literature the reactor lengths exposed to UV irradiation for the FIA determination of chlorpromazine, promethazine and perphenazine were only 50–60 cm;[25] therefore, the efficiency of UV irradiation was increased in our method. Finally, the residence times that we used were very short, in the range of 6–35 sec, against 20–45 sec for the FIA-PF determination of chlorpromazine in the literature;[27] it resulted into a greater rapidity for our method.

Interferences

Since several phenothiazine derivatives might be simultaneously present in biological samples, we evaluated the influence of other phenothiazines (acting as foreign species) on the determination of the four derivatives under study (Table 3). The experiments were performed by adding specific amounts of interferent to various concentrations of analyte. FIA-PF measurements were performed under the optimum analytical conditions defined in Table 1. The maximum concentrations of thionine, Azure A and Methylene Blue which did not interfere with phenothiazine were 8, 8 and 2 μg/ml, respectively, whereas the maximum tolerated levels of phenothiazine, Azure A and Methylene Blue were 0.25, 2 and 6 μg/ml, respectively, for the determination of thionine. Phenothiazine and Azure A were tolerated at the 0.25 μg/ml level for the determination of Methylene Blue. Finally, Azure A could be determined in the presence of maximum concentrations, of 4, 10 and 0.25 μg/ml for phenothiazine, thionine and Methylene Blue, respectively. Large differences in residence time values (see Table 1) may account for the relatively weak interferences of thionine and Azure A on the measurement of phenothiazine, and of phenothiazine and Methylene Blue on that of Azure A.

Analytical applications

In order to evaluate the analytical usefulness of the FIA-PF method, several phenothiazines were determined in real samples (human urine), using the standard addition procedure. Urine samples were diluted in water (50–200 μl urine in 25 ml water) and treated with different phenothiazines concentrations (Table 4). The fact that a good linearity was obtained for standard addition plots indicates the absence of significant interferences from compounds possibly present in human urine. Satisfactory recovery values were obtained in the ranges 97–106% for phenothiazine, 104–117% for thionine, 100–109% for Methylene Blue and 94–116% for Azure A.

Table 3. Effect of other phenothiazines on the FIA-PF determination of the phenothiazines under study*

Foreign species	Maximum tolerated level† (μg/ml) PHE‡	THI‡	MB‡	AZU‡
Phenothiazine (PHE)	—	0.25	0.25	4
Thionine (THI)	8	—	—	0.25
Methylene Blue (MB)	2	6	—	10
Azure A (AZU)	8	2	0.25	—

*Study performed under the conditions given in Table 1.

†Defined as the concentration of foreign species at which the variation of the measured PF signal is greater than ±5%.

‡Concentration = 1.0 μg/ml.

Table 4. Determination of phenothiazines in urine samples

Analyte	Concentration range (μg/ml)	Regression* equation A	B	Correlation coefficient	Recovery† (%)
Phenothiazine	0.1–1.5	1.66	0.70	0.997	97–106
Thionine	0.5–1.8	2.14	0.76	0.993	104–117
Methylene Blue	0.1–2.0	1.95	0.79	0.996	100–109
Azure A	0.3–3.0	2.19	0.73	0.987	94–116

*$\log I_f = A + B \log c$, I_f = relative fluorescence signal; c = analyte concentration (μg/ml).

†Values measured using the standard addition procedure.

CONCLUSIONS

We have developed a simple, rapid and sensitive method, combining photochemically induced fluorescence detection with FIA for the determination of neutral and charged phenothiazines. We have shown that charged phenothiazines, in spite of their relatively long optimum irradiation times in stationary media,[41] can be quantified easily and rapidly by FIA-PF.

Our satisfactory results demonstrate the possibility of using this method for routine analysis of these phenothiazine derivatives in biological samples.

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