J Fluoresc (2008) 18:1131–1138
DOI 10.1007/s10895-008-0363-9

ORIGINAL PAPER

A Spectrofluorimetric Sequential Injection Method for the Determination of Penicillamine Using Fluorescamine in the Presence of β-cyclodextrins

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Received: 6 February 2008 / Accepted: 10 March 2008 / Published online: 22 May 2008

Abstract A simple, robust and sensitive sequential injection spectrofluorimetric method for the determination of penicillamine (PA) in pharmaceutical formulations is developed. The method is based on the formation of a highly fluorescent derivative when penicillamine is reacted with fluorescamine (FL) in borate buffer of pH 9.3. The derivative produced is monitored at an emission wavelength of 495 nm using an excitation wavelength of 355 nm. The optimum conditions for the determination of PA with FL were: 3 mM FL, pH 9.3, 5 mM methyl-β-cyclodextrin, sample volume of 75 μL and reagent volume of 75 μL. Furthermore, the effect of various media on the fluorescence intensity of the PA–FL derivative was studied and methyl-β-cyclodextrin was found to give the largest enhancement. A linear dynamic range for the determination of PA of 5–80 ppm was obtained with a sampling frequency of 50 h^{-1} and a relative standard deviation of less than 2.5%. The method was applied to the determination of PA in pharmaceutical formulations with reasonable recoveries ranging from 101.0–103.1%, indicating that no interference is observed from concomitants usually present in dosage forms.

Keywords Penicillamine · Sequential injection · β-cyclodextrin · Fluorescamine · Fluorimetry

Abbreviations

SI	Sequential injection analysis
HPLC	High performance liquid chromatography
β-CD	β-cyclodextrin
Me-βCD	methyl–β-cyclodextrin
FL	fluorescamine
PA	penicillamine

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Introduction

Penicillamine (2-amino-3-mercapto-3-methylbutanic acid) is a naturally occurring sulfur-containing amino acid that belongs to the amino-thiols family. It is the main product of the decomposition of penicillin antibiotics [1, 2]. It exists in D- and L-enantiomeric forms that show different biological and toxicological properties. Penicillamine (PA) is a highly potent therapeutic agent used for many years in the treatment of various illnesses. It is used in the treatment of Wilson's disease- which results from the presence of exceedingly high concentrations of copper in the body [3].

Like most of the other common amino acids, penicillamine (PA) is not detectable by direct spectroscopic techniques as it does not posses a chromophore. Hence derivatization of this compound is essential, prior to its determination. The presence of a thiol, amino and carboxyl group in this compound provides a number of ways of interaction with organic and inorganic species that yield some spectrophotometrically active products [4–7].

Various techniques for the assay of PA have been employed, including spectrophotometry, chromatography, and titrimetry [8–13]. These methods require sample clean-up procedures, use of unstable products and use of selective detectors, which limit their use in quality control and routine clinical studies. In the United States Pharmacopoeia (USP) PA tablets are determined by an ion pairing HPLC method [14]. In this method a reversed stationary phase together with sodium-hexenesulfonate as an ion paring

reagent are used for the separation of PA with UV detection at 210 nm.

Organized assemblies, such as micelles and cyclodextrins (CDs) offer several attractive features for use in spectrochemical analyses. They are relatively cheap, non-toxic, stable, homogenous, and transparent to ultraviolet light. These media have been used extensively to alter luminescence properties of organic molecules and metal complexes [15–19]. In many cases the use of organized assemblies has led to the development of methods with better sensitivities and lower detection limits.

Cyclodextrins (CDs) are cyclic oligosaccharides build up from α-D-glucopyranose residues linked by glycosidic bonds. The most common CDs are those formed by six glucose units (α-CD), seven glucose units (β-CD) and eight glucose units (γ-CD). These compounds lack free rotation about the glycosidic bonds, thus they display a torus-like hollow truncated cone shape. The cavity of CDs is mostly hydrophobic whereas the rims, where the hydroxyl groups are located, are hydrophilic [20, 21]. The ability of such molecules to from inclusion complexes with a large number of molecules have drawn a lot of interest, especially they are considered to mimic biological systems [22, 23]. The formation of a stable complex is believed to depend on the degree of cavity size match as well as other factors such as energetic balance, the nature of the guest molecule and the degree of substitution of the CD.

In this paper we report the development of a simple, sensitive and selective SI-spectrofluorimetric method for the determination of PA in pharmaceutical preparations. The method is based on the formation of a highly fluorescent derivative when PA is mixed with FL in alkaline medium. The various experimental factors that influence the emission signal of the PA–FL derivative are systematically optimized. Furthermore, we also present the results of molecular modeling calculations to provide useful insights regarding the process of formation of PA–FL/CD inclusion complexes and the orientation of the PA–FL molecule inside the CD cavity in an attempt to understand the mechanism of the enhancement. The developed procedure was applied to the determination of PA in tablets.

Experimental

Reagents and solutions

All chemicals and solvents were used as received without further purification. Fluorescamine, Tween-20 (T20), β-cyclodextrin (βCD), DL-penicillamine, TritonX-100 (TX100), methyl-β-cyclodextrin (Me–βCD), triacetyl-β-cyclodextrin (Tri-βCD) were from Sigma-Aldrich, (Steinheim, Germany). Di-sodium tetraborate and cetyltrimethylammonium bromide (CTAB) were obtained from BDH (Poole, UK). Sodium n-dodecyl sulfate (SDS) and Tween-80 (T80) were from Kanto Chemicals (Tokyo, Japan). Hydroxypropyl-β-cyclodextrin (Hyd-βCD) was from Acros (New Jersey, USA). The water used in all experiments was Milli-Q grade. All solvents used were of HPLC-grade.

A stock standard solution of PA (500 ppm) was prepared by dissolving the pure drug in deionized water in an amber flask and kept in a dark cold place. Working solutions of PA are freshly prepared by dilutions of the stock with the appropriate solvent.

A stock solution was prepared by crushing ten tablets (Penicillamine BP, Generics, UK) and dissolving an amount equivalent to 150 mg of PA in 150 mL of deionized water with sonication at room temperature for fifteen minutes. The solution was filtered through an ordinary filter paper, washed with deionized water several times and the filtrate plus washings were diluted to the mark in a 250 mL calibrated flask.

0.01 M of the FL solution was prepared in acetone. Appropriate dilutions were prepared freshly from this solution.

A stock solution of the required βCD of 20 mM was prepared by dissolving the appropriate mass of βCD in deionized water. Dilutions with 0.01 M borate buffer of pH 9.3 were always freshly prepared.

Apparatus

The SI system (Fig. 1), FIAlab3500 (FIAlab instruments, http://www.flowinjection.com) used in this study consists of the following components: a syringe pump (2.5 ml, Sunnyvale, CA, USA), a 200 cm holding coil (0.73 mm i.d. Teflon tubing, Upchurch Scientific, Oak Harbor, USA), a multi-position-valve (eight ports, Valco, Houston, USA), a 45 cm reaction coil (0.8 mm i.d. Teflon tubing, Up-church Scientific, Oak Harbor, USA), and a Hellma (Type 176.753-QS) flow-through cell. Aminco Bowman Series-2 Luminescence Spectrometer (SLM Instrument, NY, USA) was used for the fluorescence measurements. A personal computer was used for fluid control using FIAlab for windows software (FIAlab Instruments, http://www.flowinjection.com). The SI-integrated fluidic system is connected to the computer via an RS-232C interface. The spectrofluorimeter and the data collection and evaluation were under the control of AB2 software using OS2-operating system (SLM Instrument, NY, USA). The pH values were measured using a Hanna (Romania) HI 8314-membrane pH meter.

Chemometric optimization

The SI parameters were optimized using a chemometric approach. The factors included are, sample and reagent

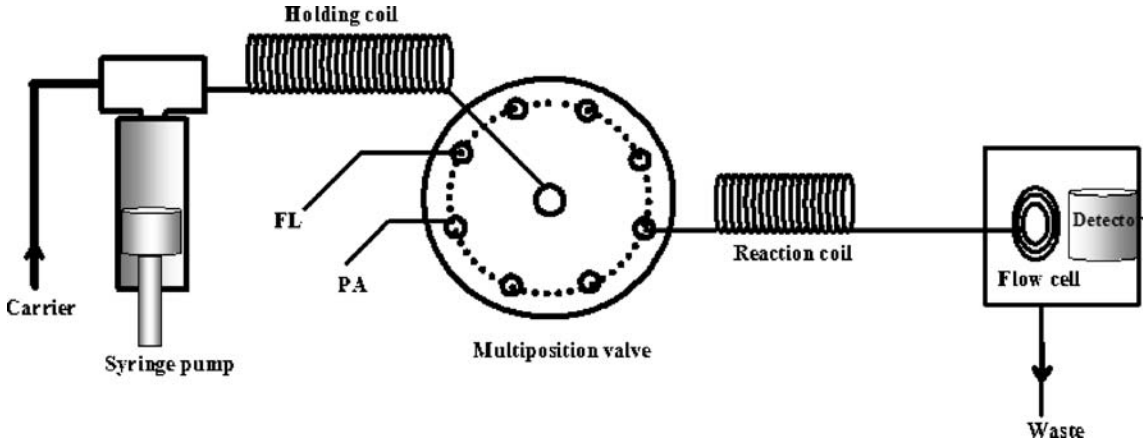


Fig. 1 Sequential injection (SI) manifold used. *FL* Fluorescamine, *PA* penicillamine

volumes and the composition of the solvent used for the preparation of FL. Use of pure acetone as a solvent for FL resulted in lowering the emission signal and in deteriorating the reproducibility due to the appearance of air bubbles when acetone is mixed with the aqueous solutions within the SI system. A 3^3 full factorial design was used for this purpose. The high and low levels adopted for this optimization are given in Table 1. The procedure was fully automated using the FIALAB-5.0 software. After implementing the design the analysis of variance (ANOVA) methods were employed to extract the factor effects. The test statistics used here is the F-ratio which is obtained by dividing the mean square of the main effect or interaction by the mean square of the error. The best model selected is the one that is significant to the *F*-test at the 95% confidence level and characterized by the highest coefficient of multiple regression.

Method

In this work, 75 μl of FL was aspirated followed by 75 μl PA standard solutions in 5 mM Me–βCD in buffer, pH 9.3 one at a time into the holding coil. A flow reversal is then used to pump the composite zone through the reaction coil and then to the detector. The fluorescence of the resultant FL–PA complex is monitored at λ_{em} 495 nm with the excitation wavelength set at 355 nm. The time required to analyze one sample is approximately 70 s. The peak fluorescence intensity was used as the performance criterion for the optimization study and for quantitative analysis.

Table 1 Levels of experimental factors used in the optimization

Factor	Level		
	Low	High	Optimum
[FL], mM	0.5	10	3.0
pH	6	11	9.3
% Acetone	30	70	50
[Me–βCD]	1	20	5
Sample volume, μL	25	75	75
Reagent volume, μL	25	100	75

Results and discussion

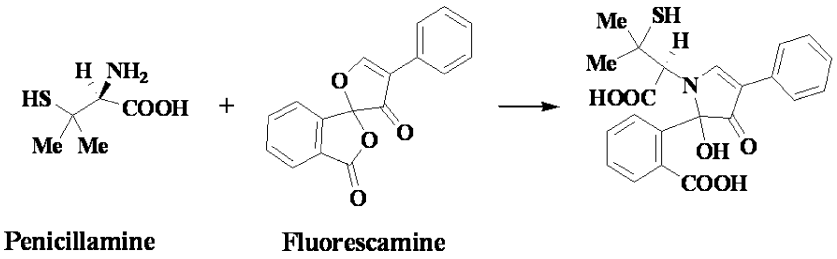
Derivatization of penicillamine with fluorescamine

Fluorescamine (FL) was chosen as a derivatizing agent because it exhibits fast reactivity towards compounds containing a primary amine group. PA contains a primary amino group, when mixed with FL in alkaline-medium, the formation of a highly fluorescent product was observed. The wavelength at which maximum emission of this product was observed is at 495 nm when excited at 350 nm. The derivatization reaction between PA and FL proceeded reasonably fast in basic media and at room temperature in less than 1 min. The reaction can be proposed to proceed as shown in Scheme 1.

In this study the factors that influence the derivatization of PA with FL, such as pH, reagent concentration and the composition of the reaction media, were investigated systematically.

The effect of pH on the reaction was studied by varying the pH of the borate buffer between 6 and 11. It is noticed that the reaction of FL with PA is strongly pH dependant. The fluorescence signal develops only in alkaline media and diminishes when the pH is lowered below 7. The emission of the derivative reached a maximum and remained almost constant in the pH range 8–9.5. Therefore, a pH of 9.3 was selected as the optimum pH and was used in all subsequent investigations.

Scheme 1 The reaction proposed between penicillamine and fluorescamine



Enhancement of the derivative emission

In an attempt to enhance the emission signal the effect of various surfactants, microemulsions, and cyclodextrins on the fluorescence of PA–FL derivative was investigated. Different surfactants solutions (0.5%), including ionic (CTAB, and SDS) and nonionic (T20, T80 and TX100) were examined. The derivative was prepared by mixing 0.6 μmol PA and 1.6 μmol FL, and then the flasks were filled with a borate buffer of pH 9.3 containing 0.5% surfactant. CTAB, T80 and TX100 enhanced the signal slightly. On the other hand, T20 and SDS resulted in a slight decrease in the emission signal.

Microemulsion solutions of SDS, CTAB and TX100, were prepared with a composition of 2:0.1:1:97 surfactant: *n*-heptane: *n*-butanol: borate buffer pH 9.3. These microemulsion solutions were used to fill the flasks containing the prepared derivatives. Again only CTAB microemulsions caused enhancement of the signal, probably due to electrostatic attraction between the positively charged micelle and the negatively charged derivative.

In this work we investigated the effect of different cyclodextrins such as βCD, Me–βCD, Hyd-βCD, and Tri-βCD, on the fluorescence of the derivative. All of the cyclodextrins studied have a significant effect on the fluorescence signal of PA–FL derivative. The effect of the various CDs on the fluorescence of PA–FL is shown in Fig. 2. In this figure a large enhancement of the PA–FL fluorescence can be clearly observed. Me–βCD gave the largest enhancement indicating a better size match of PA–FL and Me–βCD. Generally, the inclusion process results in the reduction of the rate of nonradiative deactivation processes indicating the influence of the change of the local hydrophobicity. However, the lack of spectral shift upon inclusion point out that the change of the local environment is not the only pathway that determines the enhancement of the fluorescence.

To study the stoichiometry and the association equilibrium of this complexation, series of different concentrations

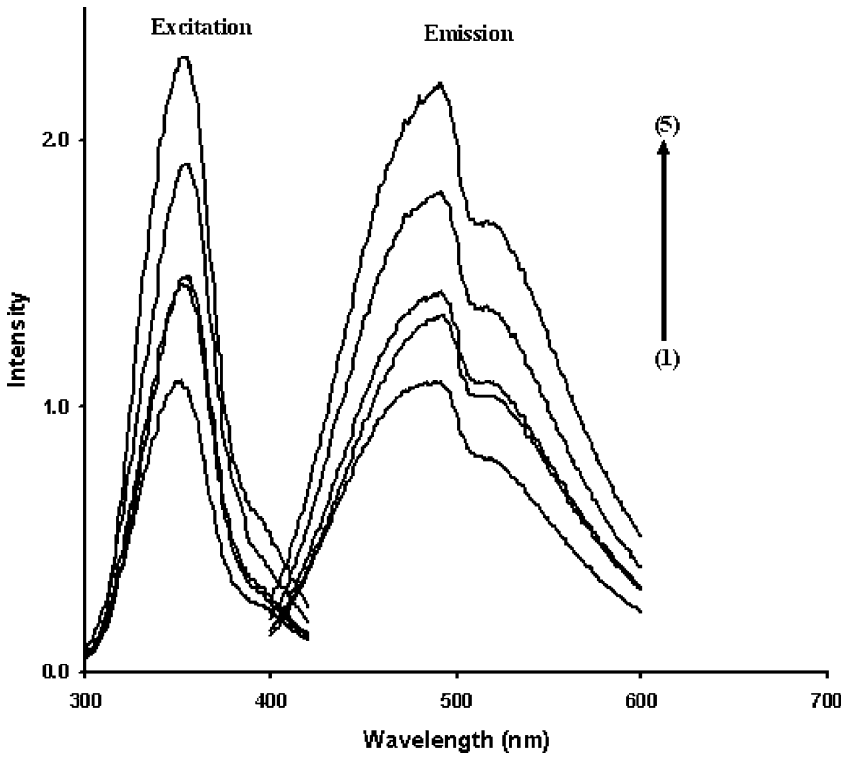


Fig. 2 Effect of different β-Cyclodextrins on the fluorescence of PA–FL derivative, [FL]=0.16 mM; [PA]= 0.068 mM; [β-CDs]=5 mM; pH=9.3; *1* Buffer, *2* Tri-βCD, *3* β-CD, *4* Hyd-βCD, *5* Me–βCD, λ_{ex}=350 nm; λ_{em}=495 nm

of Me–βCD (1–10 mM) were prepared in buffer 9.3 and used to fill the flasks that contain the derivative. The data collected in this experiment were subsequently used to determine the association constant of the inclusion complex described by the following equilibrium:

$$(\mathrm{PA}-\mathrm{FL})+\beta\mathrm{CD}\leftrightarrows[(\mathrm{PA}-\mathrm{FL})\beta\mathrm{CD}] \quad (1)$$

using a Benesi–Hildebrand approach the association constant and the stoichiometry can be obtained [18]. Assuming a stoichiometry of a 1:1, a double reciprocal plot given by the following equation is expected to produce a straight line:

$$\frac{1}{(F-F_0)}=\frac{1}{(F_\infty-F_0)K[\mathrm{CD}]}+\frac{1}{(F_\infty-F_0)} \quad (2)$$

where F_0 is the fluorescence intensity in the absence of CD, F is the fluorescence intensity at a particular concentration of CD, F_∞ is the fluorescence intensity when all of the PA–FL derivative is complexed with the CD, and K is the equilibrium constant for the 1:1 complex. A straight line was obtained when $1/F-F_o$ is plotted against 1/[Me–βCD]. It is clear from this result that FL–PA derivative forms a 1:1 inclusion complex with Me–βCD. Furthermore, a curvilinear plot of F vs. the concentration of Me–βCD was analyzed by a nonlinear regression using the following equation [24, 25].

$$F=\frac{F_o-F_\infty K[\mathrm{CD}]^n}{1+K[\mathrm{CD}]^n} \quad (3)$$

Where, n=1 for the 1:1 complex and n=2 for the 1:2 complex. The best fit was obtained for the 1:1 stoichiometry with an association constant, K, of 625±31 M^{-1}.

Molecular modeling

Molecular modeling of the inclusion complexes was carried out using the MOPAC package included in the CS Chemdraw Ultra (version 8, cambridgeSoft.com). The initial geometries of the molecule (PA–FL), βCD and Me–βCD were fully optimized using PM3 level of theory. We further constructed inclusion complexes from the PM3 optimized βCDs and PA–FL. This is performed by inserting the guest molecule in a vertical position into the βCD cavity through the primary or secondary rim of the host and perpendicular to the rim diameter. The complexation energy ΔE was defined as the difference between the sum of the energy of the individual host and guest molecule and the energy of the inclusion complex:

$$\Delta E=E_{\mathrm{complex}}-\left(E_{\beta-\mathrm{CD}}+E_{\mathrm{molecule}}\right) \quad (4)$$

Where E_{complex}, $E_{\beta-\mathrm{CD}}$, and E_{molecule} are the total energy of the complex, the free host and the free guest molecules respectively. The magnitude of the energy change would be an indicator of the driving force towards complexation. The more favorable inclusion complex is the one characterized to have the highest negative stabilization energy.

The most stable structure was obtained for the inclusion complexes where the unsubstituted phenyl ring of the guest enters through the primary rim into the cavity of βCD and Me–βCD as shown in Fig. 3 for the case of Me–βCD. PM3 calculated results for βCD, Me–βCD and their inclusion complexes are shown in Table 2. It is clear from this table that the energies of the complexes are considerably lower than the sum of the energy of the isolated guest and host and the binding energy of the complex between PA–FL and Me–βCD is lower than its complex with βCD.

The optimized βCD forms a ring with a diameter of bout 15.7 Å and a height of about 7.7 Å. Whereas, Me–βCD has a diameter of 17.6 Å and a height of 13.0 Å (the diameters and heights correspond to edges of orthorhombic box encapsulating the molecule). Therefore, PA–FL could be accommodated deeply in the cavity of Me–βCD compared to βCD. These results could explain the higher enhancement observed for the PA–FL in the presence of Me–βCD compared to βCD.

Table 2 also shows the calculated energies of HOMO and LUMO for the host, guest and the most stable complexes. It is

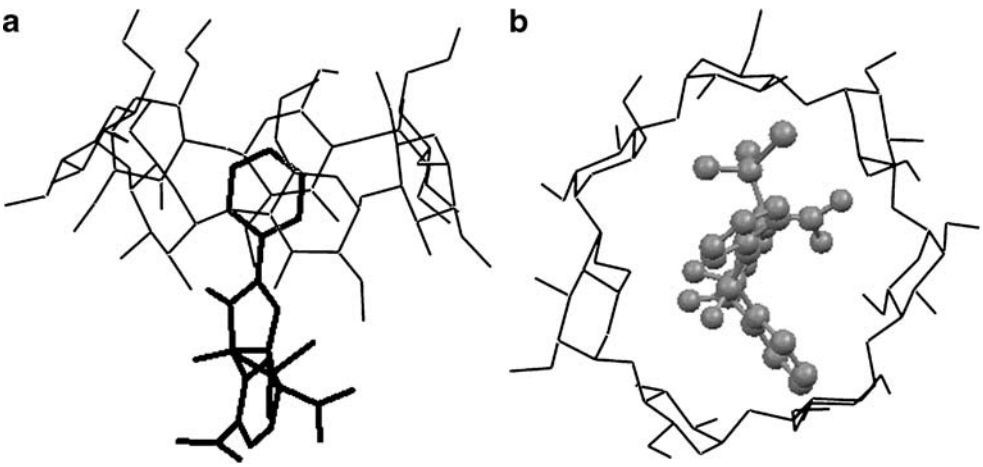


Fig. 3 Structure of the Me–βCD/PA–FL complex with the minimum energy obtained by PM3 calculation **a** seen from the side of Me–βCD wall, **b** seen from the side of the primary hydroxyl rim of the Me–βCD

Table 2 PM3 calculated parameters of PA–FL, βCD, Me–βCD, and PA–FL–CD complexes

Parameter	PA–FL	βCD	PA–FL/βCD	Me–βCD	PA–FL/Me–βCD
E (kJ/mol)	−870.3	−6,094.1	−7,026.3	−5,821.8	−6,781.6
ΔE (kJ/mol)	–	–	−61.9	–	−89.5
HOMO (eV)	−5.87	−10.48	−6.22	−10.41	−5.62
LUMO (eV)	1.973	1.37	1.48	−1.79	2.13
HOMO–LUMO gap (eV)	7.84	9.11	7.70	8.62	7.75

clear from these values that the energies of HOMO and LUMO for the complex are close to those of the free PA–FL derivative and that the HOMO–LUMO energy gap is nearly the same for the complexes and the guest molecule. This is indicative of the fact the electronic spectrum of PA–FL will not exhibit a significant change upon complexation with cyclodextrins. This is clearly shown in Fig. 2 where no significant shift was observed in the emission and the excitation spectra of PA–FL due to complexation with CDs. Obviously the complexes of PA–FL with CDs are mainly stabilized by noncovalent interactions such as van der Waals and hydrophobic forces [26].

Sequential Injection analysis

A Sequential injection (SI) method was then developed based on the conditions described above. It is known that in SI-method, lower amount of reagent and sample are consumed. This in turn will result in a decrease in the cost of the analysis especially when an expensive reagent is used. To develop the system further, we started by determining the optimum concentration of FL. various concentrations of FL (0.5–10 mM) were used to produce calibration curves for PA in the range 5–80 ppm. Our target was to obtain higher reproducible signals and calibration curves with higher slopes. Figure 4 shows the effect of changing FL concentration on the signal intensity and the slope of the calibration curve. Clearly, the highest intensity (for 40 ppm PA) was obtained at a concentration of FL of 4 mM. However, a calibration curve with a higher slope is obtained when 2 mM FL was used. Therefore, as a compromise a concentration of FL of 3 mM was used for the determination of PA. On the other hand, deionized water as a carrier produced a calibration curve with higher signals and better figures of merit compared to that obtained with a borate buffer. Therefore, deionized water was used as a carrier.

The sample and reagent volumes as well as the percent of acetone in solvents used to prepare FL are optimized using a full 3^3 factorial design. Using the factorial experimental design these three factors are changed simultaneously to produce 27 runs. The data obtained by this procedure were subjected to the ANOVA methods. From the F-test it was found that all factors, their interactions (except for the interaction of sample volume and %acetone) are significant at $P<0.05$. The value R^2 statistics indicates that the obtained model explains 98.9% of the variability of the data. Further, the polynomial model that describes the response surface was obtained. This model was used to produce response surface plots; a typical one is shown in Fig. 5 for the effect of the sample volume and the reagent volume on the intensity of the SI peak.

By inspection of the response surfaces 75 μL was found to be the optimum sample and reagent volumes. Whereas an acetone: water of 50: %50 was found optimum.

Analytical appraisal

Based on the enhancement of the fluorescence intensity of PA produced through the interaction with Me–βCD, an improved spectrofluorimetric method for the determination of PA in aqueous solution was developed. Taking into account the optimum conditions obtained (Table 1), calibration curves were produced. A linear curve was obtained for the plot of the fluorescence intensity, I, versus the

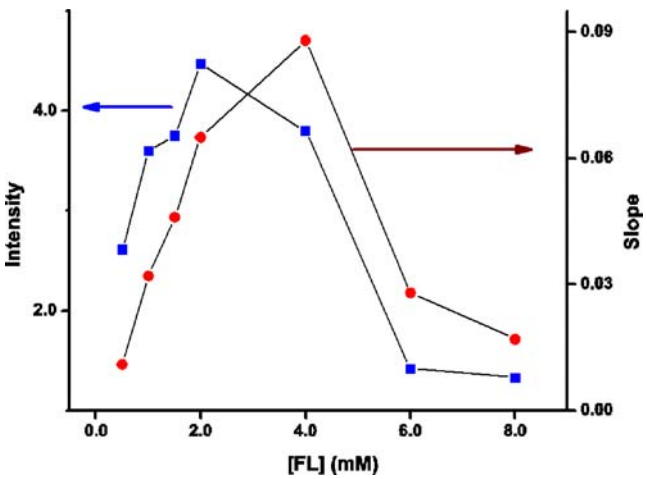


Fig. 4 Effect of Fl concentration on the signal intensity and the slope of calibration curve. -●- Effect on calibration curve slope (5–80 ppm). -■- Effect on the fluorescence intensity of 40 ppm PA

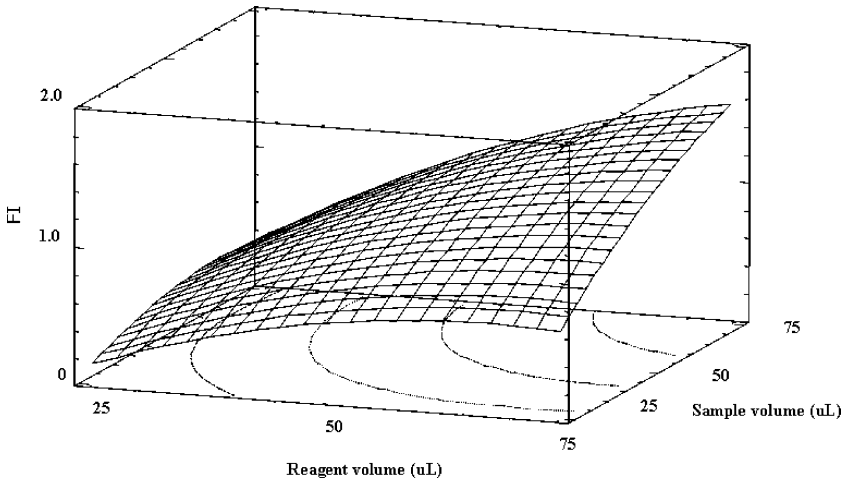


Fig. 5 A response surface plot of the effect of aspirated sample and reagent volumes on the peak emission signal

concentration of PA in ppm, C, over the range 5–80 ppm. The following calibration equation was obtained with a correlation coefficient, R^2, of 0.999:

$$I = 1.92 \pm 0.01 + (0.090 \pm 0.001)C \quad (5)$$

The relative standard deviation obtained for the standards is in the range 0.6–1.4%, indicating excellent reproducibility of the method. Also low limit of detection ($3s_b/m$; where s_b is standard deviation of the blank, and m, is the slope) of 0.1 ppm and limit of quantification ($10s_b/m$) of 0.4 ppm are obtained. Each sample was observed to require only 70 s per single run from which a sampling frequency of about 50 h^{-1} is obtained.

Application

The method was further applied to the assay of PA in commercial dosage forms such as penicillamine BP tablets using the proposed method. Each tablet of penicillamine BP contains in addition to PA, povidone, lactose, sodium, starch, glycollate, magnesium strearate, hydroxymethyl-propyl cellulose, titanium oxide, and polyethylene glycol. Table 3 summarizes the results obtained by the SI method. The method reported reasonable recoveries and the relative standard deviation values were less than 5%. The results obtained clearly suggests that the method developed is suitable for the determination of PA in pharmaceutical preparations without fear of interference usually caused by substances expected to be amongst the components of such formulations.

Furthermore, the results obtained by the SI developed method were compared with those obtained by the USP method [14]. It is clear from Table 3 that the two methods exhibit comparable accuracy and precision.

Our method on the other hand is characterized by a higher sampling frequency and uses smaller volumes of reagents and samples. Using microliter quantities of reagents and deionized water as a carrier, makes this method an environmentally friendly one and of a lower cost.

Conclusion

The development of a SI-spectrofluorimetric method has been described for the determination of PA in pharmaceutical preparations based on derivatization with fluorescamine in basic media. Me–βCD provided the greatest enhancement of the fluorescence of PA–FL, resulting in development of a method with higher sensitivity. The proposed method was applied to the analysis of PA in commercial drug samples and was found to be superior with a wide dynamic range and excellent reproducibility.

Table 3 Results obtained by the proposed SI method and the official USP [14] method for the analysis of penicillamine tablets

Drug	Active material	Mean recovery±SD (%)[a]		t[b]	F[c]
		SI-method	USP-method		
Penicillamine tablets BP (Generics, UK)	125 mg PA	101.8±1.1	101.0±0.7	1.28	2.4
Penicillamine tablets BP (Generics, UK)	250 mg PA	103.1±0.8	102.6±0.5	0.62	3.1

[a] $n=5$
[b] $t_{\text{theoretical}}$ ($p=0.05$)=2.31
[c] $F_{\text{theoretical}}$ ($p=0.05$)=6.39

The method is also simple, sensitive, consumes little amount of reagents and with a high sampling frequency.

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