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Native Fluorescence Determination of Pyridoxine Hydrochloride (Vitamin B₆) in Pharmaceutical Preparations After Sorption on Sephadex SP C-25

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ABSTRACT

A simple, batch mode, solid phase spectrofluorimetric procedure has been developed for the determination of pyridoxine hydrochloride (PY) (vitamin B₆). The method is based on the measurement of the native fluorescence of the analyte at 395 nm ($\lambda_{\text{exc}} = 295$ nm) sorbed on Sephadex SP C-25 beads. The cation-exchange gel, previously equilibrated with the sample solution, is packed in a 1-mm quartz cell in which the measurements are performed (diffuse transmitted fluorescence). The method responds linearly in the measuring range of 50–500, 10–100

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and $5\text{--}40\ \mu\text{g}\cdot\text{l}^{-1}$ with detection limits of 9.5, 2.3 and $0.60\ \mu\text{g}\cdot\text{l}^{-1}$ for 10, 25 and 50 ml of sample volume, respectively. The relative standard deviation ($n = 10$) for the determination of 100 (10 ml), 60 (25 ml) and $30\ \mu\text{g}\cdot\text{l}^{-1}$ (25 ml) of PY is 1.3%, 2.2% and 3.7%, respectively. The method, which shows increasing sensitivity as the sample volume increases, was satisfactorily applied to the determination of vitamin B₆ in pharmaceutical preparations using the procedure for 10 ml of sample.

Key Words: Pyridoxine hydrochloride; Solid phase spectrofluorimetry; Drug formulations.

INTRODUCTION

In recent years, many procedures for the determination of pyridoxine hydrochloride (PY) have been described in the literature: photometric,^[1–5] fluorimetric,^[3,6–8] chemiluminescence,^[9] high performance liquid chromatography with u.v. detection,^[4,10–13] capillary electrophoresis^[14] and gas chromatography mass spectrometry.^[15]

Fluorimetric determination of PY in pharmaceutical preparation has usually required a previous separation from the matrix^[6] or the use of zero-crossing^[3,16] and synchronous fluorescence^[7] techniques when possible. Partial least-squares multivariate calibration procedures^[8] have also been used.

Solid phase spectroscopy has been shown to be a useful and simple methodology for increasing two of the more important features of a given spectroscopic analytical procedure: sensitivity and selectivity. Thus, sensitivities as high as four orders of magnitude better than conventional methods in homogeneous solution have been reported.^[17] These two improvements are achieved by using the same instrumentation as used in the conventional procedure, based on molecular absorption^[18–20] or molecular emission.^[21]

There are two different ways of carrying out a solid phase spectroscopic method: batch mode and automatic mode. In the former, the species of interest is sorbed on an appropriate amount of a solid support by means of equilibration. The solid phase is collected and the measurements are directly performed on it.^[22–24] In the latter, the target species is measured by using an automatic continuous system in which the sample is injected and carried to the detection zone.^[2,25–27] An appropriate solid support is placed in the detection zone inside a flow cell, and the signal is developed as the sample plug reaches the solid and the species of interest is



sorbed onto it. The solid support is then regenerated to release the retained species in readiness for reutilization.^[28–30]

Analytical procedures using solid phase spectroscopy in pharmaceutical analysis have been described, most of them using the automatic mode.^[31] A few procedures in batch mode have been developed for the analysis of active principles in pharmaceuticals;^[23] nevertheless, almost all of them are spectrophotometric methods. It should be noted that there is a scarcity of solid phase spectrofluorimetric procedures in batch mode applied to pharmaceutical analysis. Recently Navalón et al. have proposed solid phase fluorimetric batch procedures for the determination of fluoroquinolones: ciprofloxacin,^[32] norfloxacin,^[33] trovafloxacin^[34] and ofloxacin.^[35]

Here, we propose a very simple batch mode, solid phase, spectrofluorimetric procedure for pyridoxine based on the measurement of its native fluorescence sorbed on a dextran type cation-exchange gel equilibrated with the sample solution. It is a selective and sensitive one step procedure; inexpensive, fast and suitable for routine control analysis of pharmaceuticals.

EXPERIMENTAL

Apparatus

Fluorescence measurements were obtained with a Perkin-Elmer LS-50 spectrofluorimeter (Beaconsfield, Buckinghamshire, U. K.) equipped with a xenon discharge lamp (20 kW), Monk-Gillieson monochromators, a Quantic Rhodamine 101 counter to correct the excitation spectra and a gated photomultiplier. The luminescence spectrometer was interfaced with a Mitac MPC 3000F-386 microcomputer supplied with FL Data Manager Software v. 2.50 for spectral acquisition. Instrument excitation and emission slits were 2.5 and 8 nm respectively and the scans were obtained at 240 nm min⁻¹. The computer was also connected to an Epson LX-800 printer for delivery of results.

All measurements were made with a quartz cell of 1 mm path length at 20°C, the temperature was controlled to within $\pm 0.1^\circ\text{C}$ with aid of a Selecta (Barcelona, Spain) Frigiterm 6000–382 ultrathermostat.

The pH measurements were made with a Crison 2000 digital pH-meter (Barcelona, Spain) furnished with a combined glass/saturated calomel electrode. The meter was calibrated with two buffers at pH 4.0 and 7.0.

An Ultrasons Selecta (Barcelona, Spain) ultrasonic bath and an Agitaser 2000 (Barcelona, Spain) rotating agitator were also used.



Reagents

Stock solutions containing $10^2 \text{ mg}\cdot\text{l}^{-1}$ of PY (Fluka, Madrid, Spain) was prepared by dissolution of the appropriate amount in deionised water. This solution is stable for four weeks stored in a refrigerator at 5°C . Working solutions were daily prepared by suitable dilution.

A succinic acid/NaOH (Merck, Darmstadt, Germany) buffer solution pH 4.0 at $C_t = 0.02 \text{ M}$ was also used.

Sephadex SP C-25 cation-exchange gel (Aldrich, Alcobendas, Madrid, Spain) was used in the H^+ form as the solid support without any pretreatment.

Fluorescence Measurements

The measured relative fluorescence intensity of the resin beads, which contained the fluorescent analyte and was packed in a 1-mm silica cell, was the diffuse transmitted fluorescence emitted from the resin at the unirradiated face of the cell. The optimum angle between the cell plane and the excitation beam was 45° ^[24] (Figure 1).

Procedure

Method for 10 ml of Sample

10 ml of sample containing between 50 and $500 \mu\text{g}\cdot\text{l}^{-1}$ of pyridoxine hydrochloride and 1 ml of buffer solution (pH = 4.0) were transferred into a 25 ml test tube with stopper, and 100 mg of Sephadex SP C-25 resin were added. The mixture was shaken mechanically for 10 min after which, the resin beads were collected from the bottom of the test tube and packed into an 1 mm cell together with a small volume of the equilibrated solution.

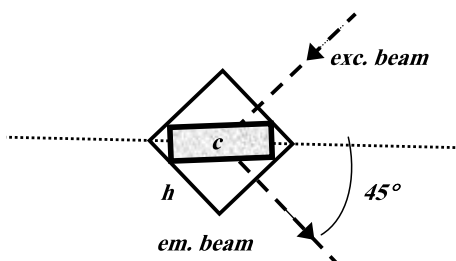


Figure 1. Arrangement for fluorescence measurements h: spectrofluorimeter cell holder c: measurement quartz cell (1 mm optical pathlength).

Then, the cell was centrifugated for 3 min at 2000 r.p.m. The fluorescence emission intensity at $20.0 \pm 0.1^\circ\text{C}$ was measured at $\lambda_{\text{em}} = 395$ nm (8 nm slit-width) using $\lambda_{\text{ex}} = 290$ nm (2.5 nm slit-width).

Every concentration used to construct the calibration line (concentration range $10\text{--}200 \mu\text{g}\cdot\text{l}^{-1}$) and the blank solution were prepared and also treated in the same way as described for the sample.

Methods for 25 and 50 ml of Sample

Operate similarly but using the indications given in Table 1. Calibration range from 10 to 100 and from 5 to 40 $\mu\text{g}\cdot\text{l}^{-1}$ for 25 and 50 ml respectively.

Treatment of Samples

Ampoules

The pharmaceutical preparations as ampoules were directly dissolved in deionised water.

Capsules

The contents of ten capsules were carefully transferred and dissolved in water by sonication. The solutions was filtered through a $0.45 \mu\text{m}$ pore size Millipore filter, and diluted to an appropriate volume with deionised water. Suitable dilutions were performed in all cases.

RESULTS AND DISCUSSION

Spectral Characteristics

Figure 2 shows the fluorescence spectra of pyridoxine hydrochloride, both in aqueous solution and on the Sephadex SP C-25 resin. No variation

Table 1. Conditions for 25 and 50 ml sample methods.

Sample volume (ml)	Volume of buffer solution (ml)	PY concentration range ($\mu\text{g}\cdot\text{l}^{-1}$)
25	2.5	10–100
50	5.0	5–40



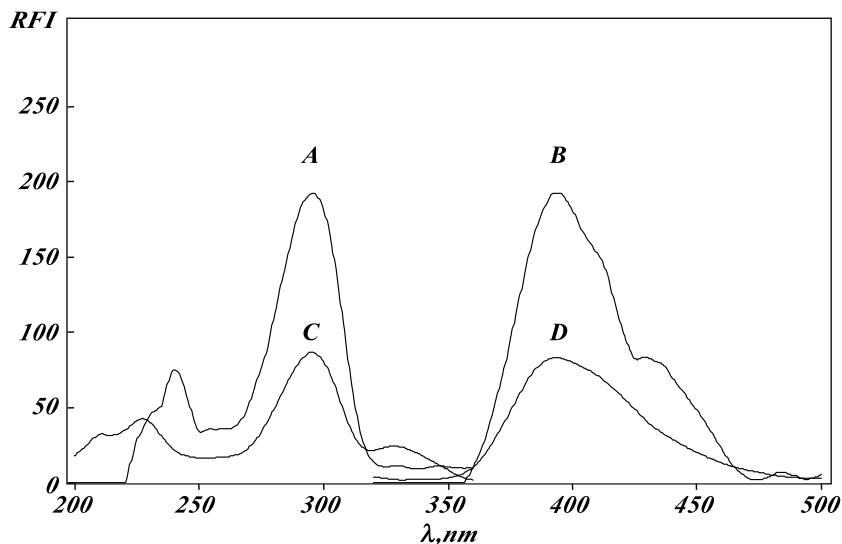


Figure 2. Fluorescence spectra (excitation and emission spectra) of PY: (A,B) equilibrated on Sephadex SP C-25 resin; (C,D) in aqueous solution. PY $200 \mu\text{g}\cdot\text{l}^{-1}$ (sample volume: 25 ml).

is observed in the peak wavelengths of both spectra in the solid phase with respect to those in aqueous solution. On the other hand, the sorption of the analyte on the solid phase resulted in a strong increase in the native fluorescence signal of the analyte with respect to that in the aqueous-only solution measured in the same cell. This is a consequence of the concentration of the analyte on the solid phase, that is, in the detection zone.

To select the optimum wavelengths, the total luminescence spectrum was registered and a contour plot was obtained from it. The maximum luminescence signal was found at 290 and 395 nm for excitation and emission wavelengths respectively.

Influence of the Nature and Amount of Solid Phase

Various ion exchangers were tested in order to retain pyridoxine hydrochloride. Anion exchangers such as Sephadex DEAE A-25 y QAE A-25 were assayed at basic pH values; cation exchangers, Sephadex SP C-25, CM C-25 and Dowex 50 w \times 8 (200–40 mesh) were tested at acid pH values. It was found that pyridoxine hydrochloride was sorbed on all solid supports tested. Sephadex SP C-25 was chosen because it offered the

highest fluorescence signal due to a more complete fixation of analyte (about 83% against 60–70% for the other supports).

The amount of solid phase used was critical because an increase resulted in a decrease in the fluorescence signal and *vice versa*. It can be explained from the equation for the fluorescence signal due to the sample species sorbed:^[18,36]

$$I_f = \phi_f I_o \varepsilon b C_R \quad (1)$$

Where:

I_f = radiant intensity of the fluorescence emission ($\text{J}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$)

ϕ_f = quantum efficiency = (n° of molecules emitting fluorescence radiation / n° of molecules excited)

I_o = radiant intensity of the excitation light ($\text{J}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$)

ε = molar absorptivity of the fluorophor on the solid support ($\text{kg}\cdot\text{mol}^{-1}\text{cm}^{-1}$)

b = optical path length (cm)

D = distribution coefficient ($\text{l}\cdot\text{kg}^{-1}$) = (*moles of analyte/kg of resin*) / (*moles of analyte/l of solution*)

C_R = concentration of sample species sorbed on the solid support ($\text{mol}\cdot\text{kg}^{-1}$)

If a volume V of sample solution (l) is equilibrated with m (kg) of ion-exchanger, the initial concentration of analyte being C_0 ($\text{mol}\cdot\text{l}^{-1}$), the concentration C_R is obtained from:

$$C_0 V = C_s V + C_R m \quad (2)$$

where C_s is the concentration of sample species left in solution ($\text{mol}\cdot\text{kg}^{-1}$). From $D = C_R/C_s$ (D = distribution ratio), it follows that:

$$C_0 V = \frac{C_R V}{D} + C_R m \quad (3)$$

$$C_R = \frac{C_0 V}{m + \frac{V}{D}} \quad (4)$$



From these equations the following expression is obtained which relates the fluorescence intensity with the mass of exchanger m (kg), the sample volume $V(l)$ and the distribution ratio D ($l \cdot kg^{-1}$):

$$I_f = \phi_f I_0 \varepsilon b \frac{C_0 V}{m + \frac{V}{D}} \quad (5)$$

$$I_f = \phi_f I_0 \varepsilon b \frac{C_0 V}{m(1 + \frac{V}{mD})} \quad (6)$$

When the value of the distribution ratio D is sufficiently large, the fraction V/D can be neglected as compared with m and we obtain the following equation:

$$I_f = \frac{KV}{m} c_0 \quad (7)$$

In fact, the graphical representation of RFI versus $1/m$ was a straight line (Figure 3) with slope = $6.2 \cdot 10^{-3}$ kg which confirms the relation of inverse proportionality between the signal of fluorescence and the amount of resin as above explained.

100 mg (10^{-4} kg) of Sephadex SP C-25 was selected as appropriate amount. This is the minimum amount needed to fill the cell up to the upper level of the excitation light beam.

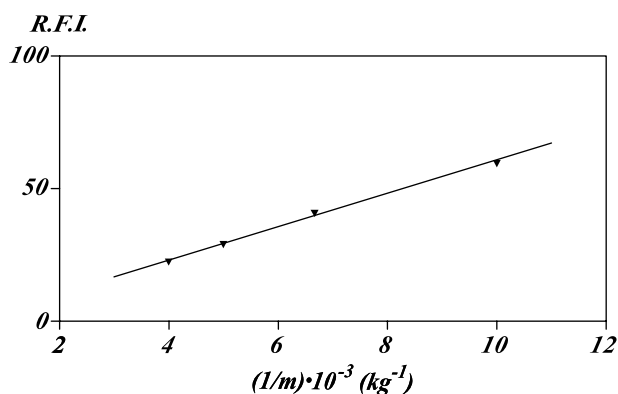


Figure 3. Influence of amount of solid phase.

Taking into account the slope, $6.2 \cdot 10^{-3} \text{ kg} = K \cdot V / C_0$ and the experimental values used for $V = 0.01 \text{ l}$ and $C_0 = 5.9 \cdot 10^{-4} \text{ mol} \cdot \text{l}^{-1}$, a value of $K = 1.05 \cdot 10^6 \text{ kg} \cdot \text{mol}^{-1}$ was found.

On the other hand, from the slope value obtained and the experimental values used for $V = 0.01 \text{ l}$ and $c_0 = 5.9 \cdot 10^{-4} \text{ mol} \cdot \text{l}^{-1}$, the value of the constant $K = \phi_f I_0 \varepsilon b$ in Eq. 2 could be evaluated.

From the slope values of the calibration graphics, KV/m ($\text{l} \cdot \mu\text{g}^{-1}$) (see Table 2), the experimental value of K could also be evaluated. For $m = 10^{-4} \text{ kg}$ and using successively $V = 0.01, 0.025$ and 0.05 l , the values obtained for K were $0.98 \cdot 10^6$, $0.91 \cdot 10^6$ and $0.81 \cdot 10^6 \text{ kg} \cdot \text{mol}^{-1}$ (mean value $K = 0.90 \cdot 10^6 \text{ kg} \cdot \text{mol}^{-1}$). This is in good agreement with the value previously obtained.

Influence of the pH of the Sample

The fluorescence emission of PY sorbed on SP-Sephadex C-25 was constant between pH 3.0 and 5.0. Above pH 5.0 the signal decreased. On the other hand, pH values between 2.0 and 3.0 produced a drastic increase in sensitivity but a poorer precision. So, pH 4.0 was selected as working pH. Several buffer solutions at pH 4.0 were tested: Biftalate/Ftalate, Acetic acid/Acetate, Succinic acid/Succinate and Citric acid/Citrate. The former gave the lower signal and the two latter, the highest; so, Succinic acid/Succinate was chosen. The increase in the buffer concentration produced a decrease in the signal, as a consequence of the competition for the active sites of the resin. A 0.02 M concentration of buffer was selected

Table 2. Analytical parameters.

Analytical parameters	Sample volumes (ml)		
	10	25	50
Linear dynamic range ($\mu\text{g} \cdot \text{l}^{-1}$)	50–500	10–100	5–40
Calibration graph Intercept	1.4	11.3	0.08
Slope ($\text{l} \cdot \mu\text{g}^{-1}$)	0.584	1.341	2.387
Correlation coefficient (r)	0.998	0.998	0.999
Detection limit ($\mu\text{g} \cdot \text{l}^{-1}$)*	9.5	2.3	0.60
Quantification limit ($\mu\text{g} \cdot \text{l}^{-1}$)**	32	7.7	2.0
R.S.D. %. (n = 10)	1.27	2.20	3.66

*(criterion 3 σ).

** (criterion 10 σ).



to obtain an appropriate buffering capacity without an excessive decrease in sensitivity.

Influence of the Stirring and Centrifugation Times

The kinetics of the fixation process proved to be fast, because the equilibration time was achieved in 10 min for all three volumes assayed (10, 25 and 50 ml of sample volume). The centrifugation of the cell before measurement did not increase the signal value. Nevertheless, the reproducibility (expressed in RSD%) was increased from 3.3% (without centrifuging) to 1.3% (for 10 ml sample procedure) when the cell was centrifuged at 2000 rpm for 3 min.

Influence of the Working Temperature

The dependence of the fluorescence intensity of the analyte on the temperature was found to be critical, as usual: with increasing temperatures in the range 0–70°C, the fluorescence intensity fell strongly, so, a working temperature was chosen of $20.0 \pm 0.1^\circ\text{C}$ and it was thermostatted. The fluorescence intensity was constant for, at least, 100 min, and no photodecomposition was observed in this time.

Distribution Measurements

Buffer solution and 100 mg of Sephadex SP C-25 resin were added to an aqueous solution containing 2.43×10^{-4} mmol of PY, and the solution (10 ml) was stirred for 10 min. The equilibrated solution was separated from the resin. Afterwards, the solution was treated in the same way with a further batch of resin (100 mg) in order to determine the concentration of PY left in the previously equilibrated solution. The distribution ratio D ($\mu\text{moles of PY sorbed per gram of resin}/\mu\text{mole of PY per ml of solution}$) was calculated from the initial and equilibrium concentration in the solution. An average value of $D = (5.3 \pm 0.3) \cdot 10^3 \text{ ml} \cdot \text{g}^{-1}$ was obtained from five replicated experiments.

Analytical Parameters

The analytical parameters for every calibration graph are summarized in Table 2. As can be seen, sensitivity, expressed as the slope of the calibration graph, increases with increased sample volume. This is due to the retention of an increasing amount of analyte on the same amount of solid phase when the sample solution volume is increased from 10 to 50 ml. This increase can be seen in Figure 4.

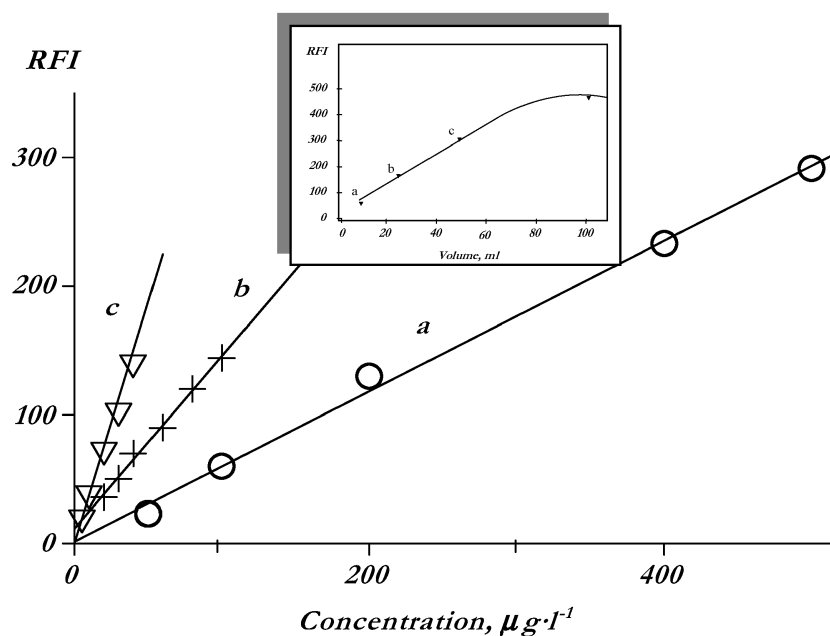


Figure 4. Calibration lines: 10 (a), 25 (b) and 50 ml (c) sample volume. Inset: Influence of sample volume on sensitivity. PY concentration: $100 \mu\text{g}\cdot\text{l}^{-1}$.

The empirical slope ratio values for each of the two procedures are: $s_{50}/s_{25} = 1.78$, $s_{25}/s_{10} = 2.30$, $s_{50}/s_{10} = 4.09$. These ratios are in good agreement with the predicted values from the theoretical Eq. 8:

$$\frac{S_{v_1}}{S_{v_2}} = \frac{V_1(mD + V_2)}{V_2(mD + V_1)} \quad (8)$$

Which give us $s_{50}/s_{25} = 1.91$, $s_{25}/s_{10} = 2.43$, $s_{50}/s_{10} = 4.66$.

Table 3. Effect of foreign species.

Foreign species	Tolerated interferent/ analyte (w/w) ratio
Vitamin B ₂	6
Glucose, saccharose	5
L-lysine, Vitamin B ₁	2
Vitamin B ₁₂ , Diclofenac Na	1
Nicotinamide	0.5



Table 4. Analytical applications.

Sample	Other species	PY labelled (mg)	mg according to the method ^a	Added concentration ($\mu\text{g}\cdot\text{l}^{-1}$)	Mean recovery concentration ($\mu\text{g}\cdot\text{l}^{-1}$)	% Recovery mean ^a \pm t σ_m
<i>Neurodavar Plus</i> (Belmac, Ltd.)	Vitamin B ₁ , ^b	50	51 \pm 2	50	49.7	99 \pm 4
	Hydroxocobal amine acetate ^c			70	69.8	99 \pm 4
<i>Antineurina</i> (Mabo-Farma, S.A., Ltd.)	Vitamin B ₁ , ^b	125	127 \pm 4	100	100.3	100 \pm 4
	Vitamin B ₁₂ , ^d			50	50.5	101 \pm 6
<i>Nervobi6n</i> (Merck, Ltd.)	Lidocaine			100	100.3	100 \pm 5
	Vitamin B ₁ , ^b	100	104 \pm 3	150	151.0	101 \pm 5
	Vitamin B ₁₂ , ^c			50	50.1	100 \pm 4
				100	101.6	101 \pm 2
				150	149.0	99 \pm 5

^aMean of three determinations.^bInterferent/analyte ratio (w/w) = 1.^cInterferent/analyte ratio (w/w) = 0.01.^dInterferent/analyte ratio (w/w) = 0.04.

The reproducibility was established for ten independent analyses of solutions containing 100, 60 and 30 $\mu\text{g}\cdot\text{l}^{-1}$ of analyte for 10, 25 and 50 ml, respectively. The detection limit was calculated by using the 3 σ -criterion^[37] and the quantification limit by using the 10 σ -criterion.^[38]

Effect of Foreign Species

A systematic study of the effect of foreign species on the determination of 100 $\mu\text{g}\cdot\text{l}^{-1}$ of PY was performed. A 10 $\text{mg}\cdot\text{l}^{-1}$ level of each potentially interfering specie was tested first. If interference occurred, the concentration was progressively reduced until interference ceased. The tolerance level was defined as the amount of foreign specie that produces an error not exceeding $\pm 5\%$ in the determination of the analyte.

The influence of 8 different species commonly found together with pyridoxine in pharmaceuticals was studied. The results are summarized in Table 3.

It can be seen that the method proposed shows a good level of tolerance to other species frequently found, together with pyridoxine in pharmaceuticals, including other vitamins of the B group (B₁₂, B₂, B₁).

Application of the Method

The proposed method was applied to the determination of PY in pharmaceutical preparations using a sample volume of 10 ml. The results obtained are summarized in Table 4.

In order to check the accuracy of the procedure, a recovery study was performed by adding three levels of known concentrations of PY, from 50 to 150 $\mu\text{g}\cdot\text{l}^{-1}$ to the pharmaceutical preparations assayed. The mean percent of recoveries (Table 4) testify to the accuracy of the procedure.

CONCLUSIONS

A solid phase spectrofluorimetric procedure in batch mode is applied to the determination of the active principle PY in pharmaceutical preparations. It is a one step procedure, extraordinarily simple, inexpensive, sensitive and selective based on the direct measurement of the intrinsic fluorescence of PY sorbed on Sephadex SP C-25. The procedure does not use derivatization reagent, so it is quicker and more inexpensive than chromatographic methods.^[10–13,15] The sorption of the analyte on the solid phase from the solution provides both a significant improvement in selectivity, because the species excluded from the resin can not interfere, and a high increase in



sensitivity. So, a detection limit as low as $0.60 \mu\text{g}\cdot\text{l}^{-1}$ can be achieved which is, much better than those provided by spectrophotometric,^[1,3] luminescence^[3,6,7,9] or even by chromatographic methods.^[4,10–13,15] In addition, good reproducibility is obtained, equal to or better than existing determination methods.^[4,7,9–11,13–15] Also, this procedure, when using 50 ml of sample volume, shows a similar or higher sensitivity than the corresponding automatic mode.^[39]

The procedure contributes to expand the number of applications of Solid Phase Spectrofluorimetry (batch mode) to pharmaceutical analysis.

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