Phosphorimetric Determination of Dipyridamole in Pharmaceutical Preparations

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Room temperature phosphorescence was applied to the determination of dipyridamole in pharmaceutical preparations. The response was linear in the concentration range 100–1600 ng ml⁻¹. The use of phosphorescence enhancers such as thallium(i) nitrate (external heavy atom), sodium dodecyl sulfate (microemulsion stabilizer) and sodium sulfite (deoxygenation agent) was studied and optimized to obtain maximum sensitivity and adequate selectivity. The determination was performed in 0.026 M sodium dodecyl sulfate, 0.0156 M thallium nitrate and 0.02 M sodium sulfite. The pH value was 11.5, adjusted by adding sodium hydroxide. The phosphorescence was totally developed in 15 min, after that the intensity was measured at λex = 303 nm and λem = 616 nm. The recovery of the method was tested on commercial formulations containing dipyridamole. The recoveries obtained were 94.67 ± 0.58% for Persantin and 96.75 ± 1.37% for Assantan.

The overall least squares regression method was applied to find the most exact straight line that fits the experimental data. The detection limit according to the error propagation theory was 16.4 ng ml⁻¹. The repeatability and relative standard deviation were also determined according to this theory.

Keywords: Dipyridamole; pharmaceuticals; phosphorimetry; room temperature phosphorescence

Phosphorimetry is a selective method for measuring different organic compounds, such as pesticides, PAHs,3 and some drugs.4 Although room temperature fluorimetry is usually more sensitive than phosphorimetry, the broad band of spectra for different compounds limits the selectivity. Therefore, phosphorimetry is used because of its better selectivity over room temperature fluorescence and absorption spectrometry.3–5 Since not all molecules that fluoresce will phosphoresce, often completely eliminating the spectral interference, and the phosphorescence is shifted to a less crowded spectral region. However, the difficulties associated with low temperature phosphorescence (LTP) make this technique unpopular. Owing to these difficulties, LTP has not been used extensively for the identification and determination of many compounds. Since its discovery in 1967 by Roth,6 room temperature phosphorescence (RTP) has attracted great interest and has become a practical technique for the detection of many organic compounds.10–18 As the sample must be adsorbed on an inert substrate such as filter-paper,19,20 the technique has the disadvantages of cumbersome sample preparation, critical drying requirements and high phosphorescent background intensity from the filter-paper substrate.

RTP can also be observed from many organic compounds in liquid solutions incorporating the phosphors into organized media such as cyclodextrins, micellar systems and microcrystalline media or by using sensitized RTP. In 1980, Cline Love and co-workers21,22 first investigated micelle-stabilized RTP and studied several PAHs. In a micellar solution the analytes included in the micellar assembly are apparently protected from the quenchers present in the solution. Observation of RTP in a micellar solution usually requires the presence of a heavy atom. It is placed as a counter ion outside the micelle, thus being in proximity to the hydrophobic molecules associated with the micelle. The high local concentration of the heavy atoms produces an efficient spin–orbit coupling that can diminish the fluorescence and increase the phosphorescence. Furthermore, the micelles can effectively screen molecules in the excited triplet state from the action of potential quenchers present in the bulk water phase. However, phosphorescence is not observed unless oxygen is removed, as it is a very efficient quencher that easily penetrates the micelles. Díaz García and Sanz-Medel23 have proposed the substitution of the troublesome deoxygenation with nitrogen by chemical deoxygenation with sodium sulfite, thus avoiding foam formation and ensuring more permanent protection of the solution against contamination with oxygen. In the presence of sulfite, however, the phosphorescence is not immediately observed. The oxygen in the bulk water phase is removed first, followed by that in the micelle on evaporation as it diffuses out. Equilibrium is achieved in a few minutes.

In the spite of the interest in observing phosphorescence in micellar solutions, only a few analytical procedures have used this technique. In this work, the appropriate experimental conditions to obtain a reproducible and maximum phosphorescence signal, when sulfite is used to eliminate the oxygen from the micellar solutions, were studied.

Dipyridamole (2,2′,2″,2′′′-[(4,8-dipiperidinopyrimino[5,4-d]pyrimidine-2,6-diyldinitriilo) tetraethano]) is shown in Fig. 1. It is an intensely yellow crystalline powder. Its solution gives a yellowish blue fluorescence. It is almost insoluble in water.

Dipyridamole is a vasodilator agent that is widely used in medicine. After oral administration, it stimulates a rise in the blood flow through the coronary circulation, providing more blood to the myocardium. This effect has been used in certain sports to increase energy production, such as via ATP molecules. Therefore, this vasodilator agent is classified, in doping terms, as a stimulant. Stimulants have been consumed in sports to increase efficiency and decrease tiredness. Stimulants include other substances that intensify the attention and could increase competitiveness and aggressiveness. Nevertheless, the uncontrolled use of such drugs could cause the loss of mental power and also have serious secondary effects that could cause grave danger to health.

Fig. 1 Structure of dipyridamole.
There have been various reports describing the spectrophotometric determination of dipyridamole in pharmaceutical tablets. In comparison with the method proposed here, these methods show low sensitivity and selectivity. The need for derivatization reactions to increase the selectivity makes these methods tedious, low accuracy is achieved and the detection limits are higher than when direct spectrophotometry is used. Only three methods have utilized the highly fluorescent nature of this drug. developed a direct spectrofluorimetric determination, the method has low sensitivity. The methods of Wilecyna-Wojulewicz and Steyn require various preliminary procedures such as extraction and separation by means of TLC. We propose the application of RTP to determine dipyridamole in pharmaceutical tablets. The main advantages of using RTP over spectrophotometric and fluorometric methods for this purpose are the high selectivity and sensitivity achieved, together with a detection limit of 20 mg l⁻¹ compared with approximately 2 µg ml⁻¹ and 100 ng ml⁻¹ for spectrophotometric and spectrofluorimetric methods, respectively.

**Experimental**

**Apparatus**

All spectrophotometric measurements were performed on an Aminco Bowman Series 2 luminescence spectrometer, connected to software which runs on the OS2 operating system. The instrument utilizes a 7 W integral pulsed xenon lamp for phosphorescence measurements. The gated photomultiplier tube detection includes a unique masking method for detection in <200 µs after the initiation of the flash lamp. Quartz glass cuvettes with pathlengths of 1.0 × 1.0 cm were used. Thermostatic equipment and a Crison Model 2001 pH meter with a glass-saturated calomel combination electrode were also used.

**Software**

The AB2 program allows the operation of the instrument to obtain excitation and emission spectra, total phosphorescence spectra and time traces, such as decay curves and time resolved curves. The Flotat program was used to generate fluorescence and phosphorescence contour spectra. Statistical analysis was performed by means of a program developed by us, which has an option menu that includes all the procedures mentioned in this paper.

**Reagents**

All experiments were performed with analytical reagent grade chemicals, pure solvents and Milli-Q-purified water. Sodium dodecyl sulfate (SDS) was obtained from Sigma (St. Louis, MO, USA) (100.0 mg dissolved 100 ml of 0.1 M SDS) was diluted to prepare working standard solutions. The stock standard solution of dipyridamole was stored and protected from the light and maintained below 5 °C. Under these conditions, it was stable for at least 2 weeks. The working standard solutions of dipyridamole were stable for at least 2 d at room temperature.

Stock standard solutions of 0.1 M SDS and 0.3 M thallium(III) nitrate were used. A 0.25 M sodium sulfite solution was prepared daily.

**Procedure**

For the preparation of the calibration graph, an aliquot of dipyridamole standard solution was pipetted into a 25 ml calibrated flask, then SDS as necessary to give a 0.026 M concentration, 2.5 ml of 0.025 M sodium hydroxide solution, 1.3 ml of 0.3 M thallium(III) nitrate solution and 2.0 ml of 0.25 M sodium sulfite were added. The solution was diluted to volume with water and shaken. After the flask had stood for 15 min in a thermostat at 20 °C, a portion of the solution was transferred into a phosphorescence cuvette and the RTP measured at an excitation wavelength of 303 nm and an emission wavelength of 616 nm.

For the analysis of Persantin (Boehringer Ingelheim, Barcelona, Spain), 10 tablets were weighed and for the analysis of Asaanin (Boehringer Ingelheim) ten capsules (which contained inside dipyridamole in the form of minute tablets) were weighed. In all instances, the solid was powdered, homogenized and about 0.1 g was taken for analysis. Suitable dilutions were made with 0.1 M SDS. In all instances, the excipients were not soluble in SDS, so after shaking and maintaining in an ultrasonic bath for 5 min the solutions had to be centrifuged.

**Results and Discussion**

**Spectral Characteristics**

Fig. 2 shows the total phosphorescence spectrum (solid line) and the total fluorescence spectrum (broken line) of dipyridamole. They are identical in form, varying only in the location of the emission wavelength of maximum intensity. In the Fig. 3(a) the phosphorescence (solid line) and fluorescence (broken line) excitation spectra at the emission wavelength of 303 nm.

![Fig. 2: Total phosphorescence spectrum (solid line) and total fluorescence spectrum (broken line) of dipyridamole.](image)

![Fig. 3: Phosphorescence (solid line) and fluorescence (dashed line) spectra of dipyridamole. Photomultiplier voltage 800 V in phosphorescence and 410 V in fluorescence. (a) Excitation spectra; and (b) emission spectra.](image)
maximum intensity are shown. Both excitation spectra are coincident, displaying two broad peaks at excitation wavelengths of 303 and 411 nm. The coincidence of the phosphorescence and fluorescence excitation spectra means an identical excitation process in both cases. This is justified in theory by the light absorption promoting an electron from the ground electronic state to the first and second excited singlets. The peak at 303 nm shows a higher luminescence intensity than that at 411 nm and the former excitation wavelength was therefore chosen for phosphorescence measurements.

Fig. 3(b) shows the phosphorescence (solid line) and fluorescence (broken line) emission spectra. The fluorescence spectrum gives a band with a characteristic emission wavelength of 488 nm, corresponding to the transition from the first excited singlet to the ground state. The phosphorescence spectrum presents a band at 616 nm corresponding to the transition from the excited triplet to the singlet ground state. The fluorescence and phosphorescence spectra show different characteristic emission wavelengths. This difference of 128 nm gives an idea of the non-radiant energy that is lost in the intersystem crossing and the subsequent vibrational relaxation to the lowest vibrational level of the excited triplet.

The phosphorescence lifetime of dipyridamole is approximately 1324 μs. This is the time required for the population of the excited triplet state to decrease to 1/e of its original value after the excitation source has been turned off. The lifetime is a means of considering the luminescence process in terms of rates. Fluorescence lifetimes are typically of the order of 1–20 ns. As phosphorescence is a spin forbidden process, phosphorescence lifetimes are considerably longer, generally ranging from milliseconds to seconds. Consequently, the phosphorescence lifetime is a measure of the forbiddenness of singlet–triplet transitions in a given molecule.

**Factors Affecting Phosphorescence**

Chemical variables were optimized to obtain maximum phosphorescence sensitivity and adequate selectivity.

Dipyridamole is almost insoluble in water, so it was necessary to use an organic solvent. Owing to the micellar properties of SDS, it was used to dissolve dipyridamole; further, the semirigid structure of the solution favours the development of phosphorescence.

The extremely high sensitivity of the triplet state to quenching by oxygen requires deoxygenation of the sample. Owing to the high efficiency of this quenching process, it is necessary to eliminate totally the oxygen in the micellar solution. The method proposed for sample deoxygenation is based on the redox reaction of sulfite with molecular oxygen to produce sulfate. It was observed that a concentration of Na₂S₂O₄ of 0.02 M was required to eliminate oxygen completely from the solutions, which was evident from the intensity of the phosphorescence signal. This decreases at higher concentrations of Na₂S₂O₄. The decrease in the signal for higher concentrations of Na₂S₂O₄ has been interpreted as the displacement of thallium(II) from the micelle because of the high concentration of sodium in the solution. An Na₂S₂O₄ concentration of 0.02 M was chosen.

The influence of pH on phosphorescence intensity was studied by adding different amounts of H₂SO₄ and NaOH to a dipyridamole solution. The phosphorescence is not significant at pH values up to 9.5. In Fig. 4 phosphorescence intensity is plotted versus the pH of the solution and, as can be readily observed, dipyridamole has maximum intensity at pH 11.5. Phosphate buffer was first used to adjust this pH value, but the phosphorescence intensity diminished. This is a quenching situation due to the formation of ecxiplexes. The explanation for this behaviour is that an excited dipyridamole molecule associates with the phosphate in the ground state. Consequently, the phosphorescence intensity of excited dipyridamole diminishes. This pH was subsequently adjusted by adding 2.5 ml of 0.025 M NaOH.

It was also necessary to establish how the phosphorescence intensity varies with changes in thallium concentration. Fig. 5(a) shows this relationship for thallium concentrations between 0.0024 and 0.0168 M. As can be readily observed, the phosphorescence intensity increases with increase in thallium concentration. For concentrations of thallium above 0.0168 M precipitation is observed. For lower concentrations of thallium, the heavy atom effect is very sensitive. When the thallium salt is not present the phosphorescence intensity disappears. A concentration of thallium of 0.0144 M gives high intensity without problems of precipitation.

The effect of SDS concentration was investigated by preparing samples with SDS concentrations between 0.014 and 0.074 M. As Fig. 5(b) shows, phosphorescence intensity of dipyridamole diminishes as the SDS concentration increases. For SDS concentrations lower than 0.014 M precipitation occurred. A 0.022 M concentration of SDS was therefore selected, giving good sensitivity while being sufficient to dissolve the dipyridamole.

From the above two experiments, the phosphorescence intensity is maximum with SDS and thallium concentrations in

**Fig. 4** Variation of phosphorescence intensity of dipyridamole with pH. The pH was varied from 9.5 to 12.5 by adding sodium hydroxide.

**Fig. 5** (a) Effect of thallium(II) concentration on phosphorescence intensity at an SDS concentration of 0.022 m; (b) effect of SDS concentration on phosphorescence intensity at a thallium(II) concentration of 0.0144 m; and (c) effect of SDS and thallium(II) on phosphorescence intensity at an SDS to thallium(II) ratio of 1.667.
the proportion of 1.667, and Fig. 5(c) shows the phosphorescence intensity versus SDS concentration with this proportion maintained. As can be observed, the phosphorescence intensity increases as the concentration of SDS increases, reaching a constant value at SDS concentrations above 0.025 M. Concentrations of SDS of 0.026 M and of thallium(i) 0.0156 M were selected as adequate. At concentrations of SDS above 0.032 M precipitation occurred.

Another factor that affects the phosphorescence intensity is temperature, the phosphorescence intensity decreasing when the temperature increased from 15 to 79 °C. This decrease is measured by plotting the relative signal increment versus temperature, i.e., the intensity at each temperature minus the intensity at the lowest temperature, divided by the intensity of the higher temperature and multiplied by 100. This relationship shows a linear behavior, the slope (which is the temperature coefficient of dipiridamole) being 1.36% °C\(^{-1}\), the intercept on the ordinate 104.4% and the coefficient of the determination \(r^2 = 0.995\).

The influence of dipiridamol concentration on the phosphorescence intensity was studied under the above conditions. The phosphorescence intensity reaches a constant value at dipiridamol concentrations above 20 \(\mu\)g ml\(^{-1}\). The dipiridamol concentration range for a linear relationship between phosphorescence intensity and concentration was found to be up to 1600 ng ml\(^{-1}\). The inner filtering effect is significant for concentrations of dipiridamol above 1600 ng ml\(^{-1}\), and increases as the dipiridamol concentration increases. Consequently, calibration was performed for dipiridamol concentrations up to 1600 ng ml\(^{-1}\) with three replicates per point.

**Determination of Dipiridamole**

Under the operating conditions outlined above, we propose a method to determine dipiridamol by direct measurement of phosphorescence intensity with an emission wavelength of 616 nm and an excitation wavelength of 303 nm in the concentration range 100–1600 ng ml\(^{-1}\). The delay time required was 120 \(\mu\)s with the photomultiplier tube masked. The gate time appropriate for this delay time was 800 \(\mu\)s and the detector voltage was 740 V. Both excitation and emission bandpasses were 16 nm and the scan rate was 5 nm s\(^{-1}\).

A calibration graph was constructed with three replicates per point. Fig. 6 shows the average emission spectra of the calibration concentrations.

The proposed method was evaluated by a statistical analysis of the experimental data by fitting the overall least squares line according to \(y = a + bx\).\(^{39,40}\) Table 1 gives the results from the statistical analysis.

The calibration line presents homoscedasticity (standard residuals have a uniform variance) and therefore it is not necessary to weight the phosphorescence intensity values according to the mean standard deviation. In order to test the linearity of the overall least squares regression, the ANOVA test was performed.\(^{39}\) The results are given in Table 2. When the 95% confidence region for true slope and intercept\(^{40}\) estimated is represented, the zero intercept on the ordinate falls within the joint confidence region. This means that the intercept on the ordinate is not significantly different from zero. Therefore, the relationship between phosphorescence intensity and dipiridamol concentration is proportional. The confidence interval for the corresponding slope is 1.257 \(\times\) 10\(^{-3}\)–1.238 \(\times\) 10\(^{-3}\). Nevertheless, the significance of the intercept was tested by applying Student’s \(t\)-test (Student’s \(t\)-test is more restrictive than the confidence region of the true slope and intercept). The experimental \(t\)-value obtained was 0.324, which is less than theoretical value of 2.12.

If the theory of error propagation is considered, the values of the detection and determination limits are consistent with the reliability of the blank measurements and the signal measurements of the standards.\(^{41,42}\) In this case a detection limit of 16.4 ng ml\(^{-1}\) and a determination limit of 54.6 ng ml\(^{-1}\) were obtained. The detection limit according to Clayton considers the probability of false positive and false negative values, the detection limit being 18.8 ng ml\(^{-1}\).

In order to study the precision of the method, a series of 10 solutions of 800 ng ml\(^{-1}\) of dipiridamole were measured on the

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**Table 1** Results from statistical analysis of data: least squares regression with replicates

<table>
<thead>
<tr>
<th>Intercept on ordinate ((a))</th>
<th>(-1.705 \times 10^{-3})</th>
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</thead>
<tbody>
<tr>
<td>SD of intercept on ordinate ((s_a))</td>
<td>(5.3 \times 10^{-3})</td>
</tr>
<tr>
<td>Slope ((b))</td>
<td>(1.249 \times 10^{-3})</td>
</tr>
<tr>
<td>SD of slope ((s_b))</td>
<td>(5.8 \times 10^{-6})</td>
</tr>
<tr>
<td>SD of regression ((s_y))</td>
<td>(1.3 \times 10^{-2})</td>
</tr>
<tr>
<td>Coefficient of determination ((r^2))</td>
<td>0.9996</td>
</tr>
<tr>
<td>Confidence interval of intercept on ordinate</td>
<td>(9.447 \times 10^{-3})–(-1.286 \times 10^{-3})</td>
</tr>
<tr>
<td>Confidence interval of slope</td>
<td>(1.262 \times 10^{-3})–(1.237 \times 10^{-3})</td>
</tr>
<tr>
<td>Slope without intercept on ordinate</td>
<td>(1.248 \times 10^{-3})</td>
</tr>
</tbody>
</table>

**Table 2** ANOVA test: linearity test

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>SS</th>
<th>DF</th>
<th>MS</th>
<th>(F_{\text{exp.}})</th>
<th>(F_{\text{theoretical}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Due to regression</td>
<td>8.2804</td>
<td>1</td>
<td>8.2804</td>
<td>2.6916 (\times) 10(^{-4})</td>
<td>4.67290 (\times) 10(^{-5})</td>
</tr>
<tr>
<td>Total within line (lack of fit)</td>
<td>6.2636 (\times) 10(^{-3})</td>
<td>12</td>
<td>2.1969 (\times) 10(^{-4})</td>
<td>3.26</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8.2833</td>
<td>17</td>
<td>0.4873</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* SS = Sum of squares of deviation; DF = degrees of freedom; MS = \(\frac{SS}{DF}\).
same day. By applying the IUPAC definition, the mean standard deviation of replicates was 3.88 ng ml⁻¹ and the relative error 1.1%, whereas if error propagation is assumed, the SE obtained was 2.59 ng ml⁻¹ and the relative error 0.71% (95% confidence level). In this case the relative error for replicates is less on applying the SE of the regression line, owing to the classical hyperbolic shape of the confidence and dispersion bands of the true line which are closest at the mean concentration, this being very near to 800 ng ml⁻¹, where the standard deviation is reduced to the standard deviation of the mean, and therefore, the relative error is less. To estimate concentration values at greater distances from the mean concentration of the regression line, the error propagation method gives the greatest values for the relative error.

The accuracy of the method was established by testing the analytical signal corresponding to the concentrations of the calibration line. As can be seen from the RSDs in Table 3, the values obtained by applying the error propagation theory confirm that, to calculate the confidence interval of a measurement, the error propagation method may be used.

The determination of dipyridamole by RTP was compared with the currently accepted method. In this, 1 ml of the problem solution is extracted at pH 10 with 8 ml of hexane–isoamyl alcohol (95 + 5) and the organic phase used directly for measurement of the fluorescence intensity (λex = 405 and λem = 495 nm). A comparison study of 10 samples of dipyridamole was performed by applying least squares paired analysis with the concentration calculated in reverse by means of the currently accepted method and the proposed method. As both methods should give the same concentrations for the same samples, a zero intercept on the ordinate and a slope of units must be obtained. Fig. 7 shows the 95% confidence region for the true slope and intercept estimated. As can be observed, the point corresponding to the zero intercept and unit slope falls within the joint confidence region. This means that the intercept is not significantly different from zero and the slope is not significantly different from unity. Consequently, the accuracies of proposed and currently accepted method are not significantly different.

**Applications and Interference Study**

The recommended procedure was applied satisfactorily to the determination of dipyridamole in the Spanish pharmaceutical products that contain this vasodilator agent in different proportions (Persantin 100 mg and Asasantin 75 mg). The assay results expressed as a percentage of the nominal contents resulting from the average of three determinations of the different tablets were 94.57% for Persantin and 96.75% for Asasantin, the standard deviations being 0.58% and 1.37%, respectively. The recoveries agree well enough with the nominal content and the precision is quite satisfactory.

The specificity of this determination was studied by adding selected drugs to the pharmaceutical preparation of dipyridamole and other drugs with intrinsic fluorescence and testing to see if the added drugs caused interference in the dipyridamole quantification. In the first case, dipyridamole (1600 ng ml⁻¹) was spiked with saccharose, lactose and glucose (20 μg ml⁻¹). No interferences were found. In the second, the added drugs were aspirin, aminophen, spironolactone and cromene acid (1 μg ml⁻¹), amiloride, furosemide and triateren (25 ng ml⁻¹), aspirin and nabolol (2 μg ml⁻¹), metropol (80 ng ml⁻¹) and caffeine, quinidine and quinic (1.6 μg ml⁻¹). Except for the furosemide, no significant variation of the analytical signal was observed from the value expected when dipyridamole is present alone. Nevertheless, interferences were observed if the concentrations of these drugs were increased.

**Conclusions**

A method for direct phosphorimetric determination of the vasodilator agent dipyridamole in pharmaceutical preparations has been developed. The determination can be performed by measuring phosphorescence intensity within an emission wavelength of 616 nm after excitation at 313 nm with excellent repeatability and sensitivity.

Owing to the high selectivity of the phosphorimetric methods, the determination of dipyridamole by RTP shows no significant interferences and is suitable for its determination in tablets. Other components of the tablets do not interfere in the phosphorescence spectra of dipyridamole.

An exhaustive statistical analysis was applied to the calibration graph, including least squares regression and ANOVA. The regression line shows homoscedasticity. The validity of the overall least squares regression is proved by the ANOVA test, the variation of group means about the line, which means the lack of fit, not being significantly different from the variation within groups (pure error). Therefore, the model chosen is an adequate description of the true relationship between phosphorescence intensity and dipyridamole concentration.

The proposed method was compared with the currently accepted method and the accuracies were not significantly different.

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**References**
