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Artigo

HEAVY ATOM ENHANCED ROOM-TEMPERATURE PHOSPHORIMETRY FOR ULTRATRACE DETERMINATION OF HARMANE

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Harmane has been proposed for the treatment of epilepsy, AIDS and leshmaniosis. Its room-temperature phosphorescence was induced using either $AgNO_3$ or $TlNO_3$, enabling absolute limits of detection of 0.12 and 2.4 ng respectively, with linear dynamic ranges extending up to 456 ng ($AgNO_3$) and 911 ng ($TlNO_3$). Relative standard deviations around 3% were observed for substrates containing 46 ng of harmane. Such sensitivity and precision are needed because harmane intake must be strictly controlled to achieve proper therapeutic response. Interference studies were performed using thalidomide, reserpine and yohimbine. Recovery of 104±6% was achieved using solid surface room-temperature phosphorimetry. The result was comparable to the one obtained by micellar electrokinetic chromatography.

Keywords: room-temperature phosphorescence; harmane; selective heavy atom effect.

INTRODUCTION

Instead of a single disease, epilepsy has been defined as a cluster of neurological disorders that has been focus of several studies in order to better understanding it[1]. Recent studies have demonstrated that β-carboline alkaloids may have strong effect on the threshold for inducing of epileptic seizures. Harmane (1-methyl β-carboline) (Figure 1) has prevented electroshock induced seizures in a dependent-way dose. Increasing intakes of harmane has decreased the frequency of convulsions from 66% (using 2.5 mg of harmane per kg) to 20% (10.0 mg of harmane per kg). However, the same study showed that chemically induced seizures (by the use of pentylenetetrazol) are decreased with the low intake of harmane (2.5 mg per kg) while aggravated with the high dose of the drug (10 mg per kg). This study has demonstrated the potential of harmane as a drug for the treatment of epilepsy if the drug intake is strictly controlled[2].

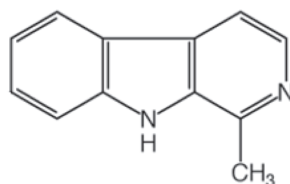

Figure 1. *Harmane (1-methyl β-carboline)*

β-carbolines have also other biological activities, acting as antimicrobial and hypotensive. They also have hallucinogenic and antioxidative properties[3,4]. Although harmane has not yet been used as the active principle of any commercial medicine, studies have indicated that this substance has antileishmanial and anti-HIV activity[5-7].

Harmane is the main β-carboline alkaloid that occurs naturally in plants such as *Peganum harmala* and in other plant species throughout the world[8]. It is also found as a product of pyrolysis of indoles and proteins and thus occurs in cigarette smoke, charcoal grilled meats and teas[9-11]. Harmane can also be endogenously produced in human and animal tissue as a product of secondary metabolism[12], however, most generally, the presence of harmane and other β-carbolines in human body is from exogenous sources[13].

Luminescence of harmane has been studied at 77 K[14-16], however, no information on room-temperature phosphorescence and no studies concerning the influence of heavy atoms as enhancers of the phosphorescence signal have been reported. Solid surface room-temperature phosphorimetry (SSRTP) has been established as a selective analytical technique, allowing ultratrace determination of many organic substances of environmental, clinical and biological interest, in special for alkaloids[17,18]. Phosphorescence of analytes of interest can be selectively induced in the presence of potential interferents by the right choice of the experimental conditions, in special, the use of selective heavy atom enhancers and the adjustment of the pH of the analyte solution to be spotted on the substrate[17]. The presence of heavy atoms in the vicinity of a molecule may induce or enhance its phosphorescence due to the increasing of the spin-orbit coupling interaction. This phenomenon may affect singlet-triplet transitions and either radiative or non-radiative decay from the excited triplet state, therefore, an increase in phosphorescence quantum yield does not always occur, being the selective nature of the external heavy atom effect based on these different interactions between analytes and heavy atoms[17]. The control of the pH of the analyte solution is a very important factor since variations of the pH may produce derivatives with larger phosphorescence quantum yield. Moreover, charged species formed in the solution may present higher phosphorescence owing to the fact that ionized species have greater molecular rigidity when adsorbed on the cellulose substrate[19].

SSRTP may enable selective determination of harmane in the presence of other concominat substances (for instance, zidovudine, thalidomide, reserpine and yohimbine) without the need for previous separations. This feature may impose great advantages in terms of labor and operational cost when compared to HPLC based methodologies[20,21]. Because of its potential clinical applications and the dose-dependent response, the development of a simple and sensitive analytical method for the quality control of harmane based drugs is desirable. In the present work, SSRTP is evaluated as a

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tool for the determination of harmane in simulated pharmaceutical tablets. The goal is to achieve selective conditions to allow determination of harmane in such samples without the need for previous separation of other components in the sample.

EXPERIMENTAL

Instrumentation

Phosphorescence was measured on an LS-55 Perkin-Elmer spectrofluorimeter (Perkin-Elmer, Norwalk, USA). This instrument was equipped with a pulsed xenon arc lamp as the excitation source, two 1200 groves/mm diffraction gratings and a R928 photomultiplier detector. Phosphorescence was acquired using 3 ms delay and 3 ms gate times. Such conditions eliminated second order scattering and fluorescence background from samples. Spectral bandpass was set to 10 nm. An adapted front surface accessory (Perkin-Elmer) was used to place the cellulose substrates for the measurements. This apparatus was modified to enable nitrogen purging directly onto the sample during measurements. Micellar electrokinetic chromatography (MEKC) determination was made on a capillary electrophoresis system (Agilent, USA).

Photochemical treatment for paper background reduction were carried out in a laboratory made photochemical reactor loaded with six mercury sterilization lamps (6 W each one with maximum emission wavelength of the lamps at 254 nm) placed on the top of the reactor.

Reagents

Filter paper (Whatman No. 42) treated in the laboratory for reduction of background was employed as a sample substrate. Deionized water (resistivity of 18.2 MΩcm) from a water ultra purifier master system 1000 (Gehaka, Brazil), was used to prepare all aqueous solutions. Nitrogen (99.996 %) was from White Martins, Brazil and it was further purified passing it through an ammonium metavanadate solution and dried in a silica gel bed. All reagents were of analytical grade and they were employed without further purification. Methanol, acetonitrile, urea, mercury (II) chloride, potassium iodide, lead (II) nitrate, sodium nitrate, sodium dodecil sulfate (SDS), boric acid, acetic acid, phosphoric acid, hydrochloric acid and sodium hydroxide were purchased from Merck (Brazil). Harmane, reserpine and yohimbine were provided by Sigma (USA). Thallium (I) nitrate and cadmium nitrate were from Across (USA). Thalidomide and zidovudine were donated by Microbiológica, Brazil. A formulation containing thalidomide (Talidomida CEME®, Brazil) was used to simulate a harmane-associated medicine.

Procedures

The procedure for paper substrate background reduction was adapted from the traditional one[18] in order to decrease its overall time from 16 to 4 h. The procedure consisted on submitting filter paper strips to a hot boiling washing step (2 h in a Sohxlet extractor). After dried under an infrared lamp and cut in circles of 18 mm of diameter, in order to fit in the solid substrate cell, these substrates were then treated with UV radiation (2 h) in a photochemical reactor. This procedure reduced background from cellulose down to 95%.

Methanol was employed to prepare harmane stock solutions. Analyte standard solutions were prepared by sequential dilutions of the stock solution with methanol/water 25/75%, v/v. The pH of the analyte solutions was adjusted by adding either strong base or acid aqueous solutions or by using Britton-Robinson buffer (0.04 mol L^{-1}). Deionized water was used to prepare all heavy atom salts and SDS solutions. The concentration of these solutions for the initial studies were KI (1.0 mol L^{-1}) $TlNO_3$ (0.5 mol L^{-1}), $Pb(NO_3)_2$ (0.5 mol L^{-1}), $Cd(NO_3)_2$ (0.25 mol L^{-1}), Hg_2Cl_2 (0.25 mol L^{-1}), $AgNO_3$ (0.01 mol L^{-1}) and SDS (0.07 mol L^{-1}).

Five µL of analyte standard solutions or sample solutions were deposited on the paper substrate, using an adjustable micropipette. When necessary, the substrates were previously spotted with 5 µL of surfactant (SDS) followed by 5 µL the heavy atom solution. Then, the substrates with samples and blanks were dried at room temperature in a vacuum desiccator for approximately 2 h. The desiccator was covered with aluminum foil in order to avoid possible effects from the ambient light. Three minutes prior to and during the phosphorescence measurement, a dry nitrogen flow was passed over the substrate surface to minimize quenching effects from oxygen and air moisture.

Laboratory made tablets were prepared by mixing 60 mg of harmane with a pulverized pharmaceutical tablet containing 100 mg of thalidomide. This mixing was performed in a mechanical shaker for 1 h. Six weighted portions of the powder were then separated and washed with methanol to dissolve harmane. The solution was vacuum filtered and the filtered solution was then diluted in methanol/Britton-Robinson buffer (pH 11.0) 25/75%, v/v, when $AgNO_3$ was used as phosphorescence enhancer, or in methanol/water 25/75%, v/v when the phosphorescence enhancer employed was $TlNO_3$.

The following conditions were used for MEKC: uncoated silica capillary (50 cm effective length and 56 µm internal diameter); applied potential of 25 kV; temperature of 30 ºC; injection made at 50 mbar for 10 s. The MECK buffer was freshly prepared and typically consisted of 20 mmol L^{-1} borate buffer (pH 9.0) containing SDS (50 mmol L^{-1}), urea (0.6 mol L^{-1}) and acetonitrile 15%, in volume. The absorption of the analyte peak was detected at 254 nm. This procedure was adapted from Cheng and Mitchelson[22].

RESULTS AND DISCUSSION

RTP characteristics of harmane

Room-temperature phosphorescence (RTP) of harmane was studied in three different conditions using several heavy atom salts as potential signal enhancers. First, harmane (4 x 10^{-4} mol L^{-1}) solutions were prepared dissolving the analyte in methanol/water (25/75% v/v) before spotting it on the cellulose substrate. Alternatively, previous to the deposition on the substrate, harmane solutions were prepared in acid or in basic conditions using respectively methanol/0.2 mol L^{-1} HCl solution (25/75% v/v) or methanol/0.2 mol L^{-1} NaOH solution (25/75% v/v).

While no phosphorescence was observed in basic conditions, a small phosphorescence from harmane was observed either from the methanol/water solution with the maximum excitation and emission wavelength pair ($\lambda_{exc}/\lambda_{em}$) at 250/495 nm or from acid conditions at 250/504 nm.

The effect of six heavy atom salts on the RTP characteristics of harmane was studied. From neither basic solution (NaOH 0.2 mol L^{-1}) nor acid solution (HCl 0.2 mol L^{-1}), significant harmane phosphorescence was observed in the presence of KI, $TlNO_3$, $AgNO_3$, $Cd(NO_3)_2$, Hg_2Cl_2 and $Pb(NO_3)_2$. For harmane prepared in methanol/water solution, very intense phosphorescence is observed when it was placed on paper substrates containing either $TlNO_3$ or $AgNO_3$ with signal amplification of 34 and 164 times respectively when compared to the phosphorescence from harmane in the absence of these phosphorescence enhancers. The phosphorescence spectra under

these conditions are shown in Figure 2. No relevant phosphorescence was observed in the presence of the other four heavy atom salts.

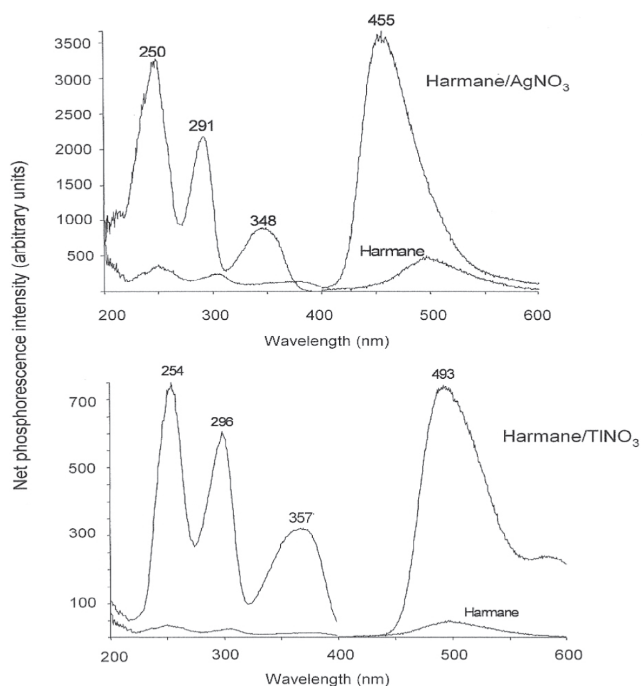

***Figure 2.** Room-temperature phosphorescence of harmane (4 x 10^{-4} mol L^{-1})*

Maximization of the harmane RTP signal

Studies to maximize the RTP signal were performed using either $AgNO_3$ or $TlNO_3$ in the substrate. A more specific study to evaluate the influence of the pH (Britton-Robinson buffer with pH values between 2 and 12) of the analyte solution to be spotted on the substrate was also performed. In substrates containing $TlNO_3$, no significant variation of signal was observed between pH 2.0 and pH 10.0. For solutions of higher pH values, a decreasing of signal was observed. Therefore, no Britton-Robinson buffer was used in the harmane solutions to be deposited on substrates containing $TlNO_3$. For substrates containing $AgNO_3$, the best signals were found from analyte solutions prepared at pH 11.0 as illustrated in Figure 3.

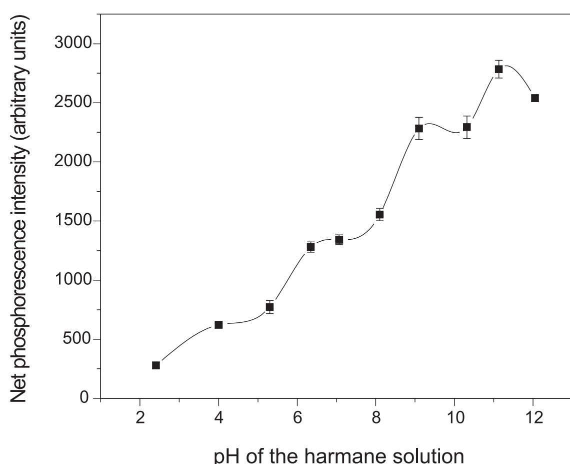

***Figure 3.** Influence of the pH of the harmane solution on its room-temperature phosphorescence in cellulose substrates in the presence of $AgNO_3$*

The concentrations of the heavy atom solutions deposited in the cellulose substrate were optimized. For $AgNO_3$, solutions with lower concentrations deposited in the substrate enabled better results, as can be seen in Figure 4a. A decreasing of harmane signal was observed for higher amounts of $AgNO_3$ due to a pre-filter effect caused by the formation of a dark film on the surface of the substrates spotted with silver solutions. For $AgNO_3$, the 0.01 mol L^{-1} solution (5.4 µg of silver for 5 µL solution volume) was selected for further experiments. In the case of $TlNO_3$, as the amount of $TlNO_3$ deposited in the cellulose substrate is increased, more intense RTP from harmane was obtained (Figure 4b). The 0.25 mol L^{-1} $TlNO_3$ solution (255 µg of thallium for 5 µL solution volume) was then selected. Higher concentrations of $TlNO_3$ were not tested because of limitations on the solubility of the salt and also because the single addition of solutions onto substrates was the chosen approach.

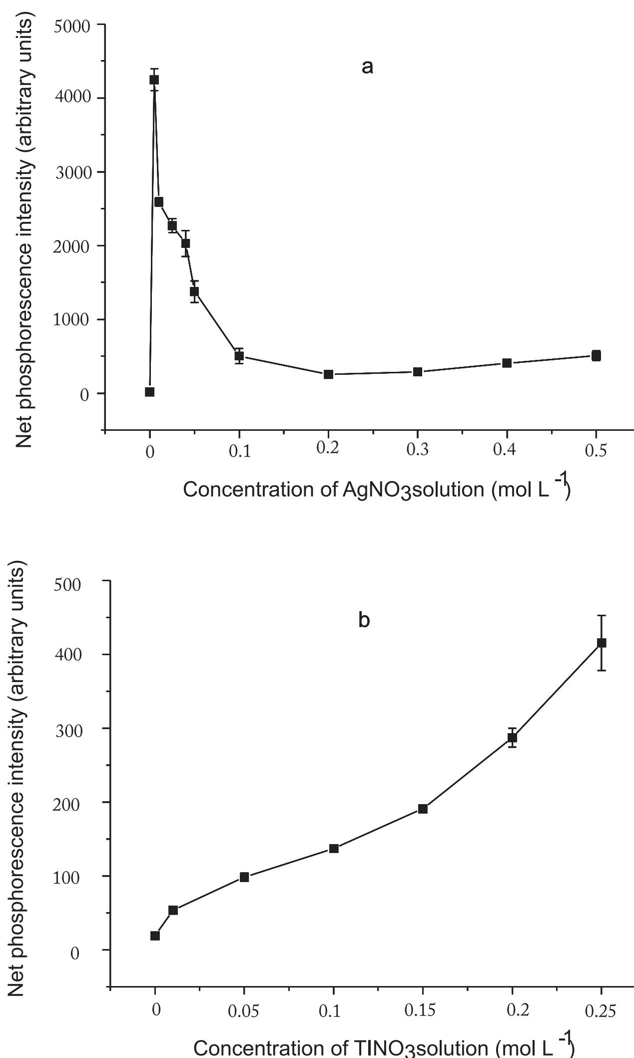

***Figure 4.** Phosphorescence of harmane in function of the concentration of the heavy atom enhancer solution spotted on the substrate: (a) $AgNO_3$ and (b) $TlNO_3$*

Sodium dodecil sulfate (SDS) is known to improve the interaction between the analyte and the heavy atom with consequent enhancement of RTP intensity. The influence of increasing SDS quantities on the substrate was studied. For $AgNO_3$, the use of SDS decreased the harmane RTP while no influence was observed in the case of $TlNO_3$. After dried on the surface of the substrate, SDS

forms a supporting layer that may help to improve analyte rigidity and/or enable better contact between analyte and heavy atom. However, depending on the analyte, such improvements due to the presence of SDS are not observed[18].

Analytical figures of merit

Analytical figures of merit were estimated using two different experimental conditions (Table 1), taken analytical curves for which each of the RTP intensities plotted was an average of four replicates. For RTP enhanced by $AgNO_3$, a linear range (described by the following equation: $Y = 3.26 \times 10^5X + 79$, where the sensitivity of the curve is displayed in ng^{-1} and the linear coefficient is in arbitrary units of phosphorescence) was observed from the absolute limit of quantification (0.39 ng) up to 456 ng of harmane. The r^2 value for this response was 0.9949. When $TlNO_3$ was used as the phosphorescence enhancer, the linear response of the RTP signal extended from 8.1 ng (absolute limit of quantification) to 911 ng ($Y = 3.73 \times 10^4X + 44$, $r^2 = 0.9815$). For sample volumes of 5 µL, the absolute limits of detection of 0.12 and 2.4 ng were obtained in the presence of $AgNO_3$ and $TlNO_3$ respectively. Both, absolute limits of detection (ALOD) and absolute limit of quantification (ALOQ) were calculated using: $kS_b/m \times 182.2\ g\ mol^{-1} \times 5 \times 10^{-6}\ L$, where S_b was the standard deviation of 16 blank measurements, m is the sensitivity of the curve in L mol^{-1} and k is the confidence value (3 for ALOD and 10 for ALOQ). Blank signals in the presence of $TlNO_3$ were about three times more intense than the blank signals observed in the presence of $AgNO_3$. The standard deviations of these two different blanks were similar. Precision studies (repeatability) indicated a standard deviation of less than 3% for 10 successive measurements of 46 ng of harmane (5 µL of a solution 1×10^{-5} mol L^{-1}) placed in substrates containing either of the phosphorescence enhancers.

Table 1. Resume of the experimental and instrumental conditions optimized for the determination of harmane

Parameter	Phosphorescence enhancer	
	$AgNO_3$	$TlNO_3$
$\lambda_{exc}/\lambda_{em}$	250/455 nm	254/493 nm
Phosphorescence enhancer concentration (effective mass)[a]	0.01 mol L^{-1} (5.4 µg)	0.25 mol L^{-1} (255 µg)
Solvent system	Methanol/ Britton-Robinson buffer (pH 11) 25/75%, v/v	Methanol/buffer 25/75%, v/v

[a]Effective mass of silver or thallium in the substrate considering 5 µL of solution.

Selectivity study

The influence of four substances that could interfere in the determination of harmane was evaluated. Three of the chosen substances are naturally fluorescent and impose serious spectral interferences when fluorescence of harmane is measured without previous separation of components, for instance, by HPLC. The interferents were separated in two groups. The first one contain two substances (thalidomide and zidovudine) that could be used as a complementary or associated active substances in anti-HIV pharmaceutical preparations, since recent studies have indicated harmane as a potential drug for AIDS treatment[7]. Thalidomide presents natural fluorescence in a spectral range close to the one presented by harmane. Neither zidovudine nor thalidomide presented any phosphorescence signal under the experimental conditions optimized for harmane. Therefore, no spectral interferences are expected. In addition, by measuring RTP signals from harmane in mixtures containing either zidovudine or thalidomide in amounts up to ten times higher, no significant differences were found in comparison to the results observed from substrates containing only the equivalent amount of harmane. Such behavior indicated the absence of matrix interferences from these substances.

The second group of potential interferents included the indolic alkaloids reserpine and yohimbine that are therapeutically used as monoamine oxidase inhibitors having specific protective effect against seizure susceptibility. For the indolic alkaloids, when $TlNO_3$ was used as the phosphorescence enhancer, intense RTP is observed from yohimbine in the same spectral region of harmane[23]. Therefore, a spectral interference is expected. When $AgNO_3$ was used as the phosphorescence enhancer, no RTP was observed for any of these two indolic alkaloids when spotted on the substrate from solutions at pH 11.0. Further studies indicated no decreasing of the harmane RTP signal when mixed in solution with each one of these indolic alkaloids in molar concentrations up to fifty times higher. Therefore, a selective determination of harmane in the presence of reserpine or yohimbine may be performed without previous separation of components.

Determination of harmane in a simulated pharmaceutical tablet

Since pharmaceutical formulations containing harmane were not available, the SSRTP method was tested by analyzing a laboratory simulated tablet. This tablet was prepared as an association of harmane and thalidomide. This was made by pulverizing separately three commercial tablets (Talidomida CEME®) containing an equivalent amount of 100 mg of thalidomide and mixing to each of the resultant powder to 60 mg of harmane. These three portions of powder were treated with methanol to dissolve the analyte, and the resultant solutions were gently filtered under vacuum. Aliquots of these solutions were collected and diluted in methanol/Britton-Robinson buffer (pH 11.0) 25/75% v/v to be quantified using the analytical curve procedure and substrates containing $AgNO_3$ as phosphorescence enhancer. The same quantification procedure was made using substrates containing $TlNO_3$, however, in this case, the sample was diluted in methanol/water 25/75% v/v previously to the deposition on the substrate. A mean recovery of 104 ± 6% (n=6, three for each heavy atom salt), was achieved, indicating the suitability of such application. These same laboratory simulated tablets were analyzed by micellar electrokinetic chromatography (MEKC) in order to compare to the recoveries achieved using SSRTP. The mean recovery of 96±6% (n=5) was obtained using MEKC. A statistical test indicated no difference in the results achieved by the two methods ($p=0.05$; $n_{SSRTP}=6$; $n_{MEKC}=5$). Figure 5 shows $TlNO_3$ enhanced RTP spectrum and electropherogram for thalidomide based formulation in the presence and in the absence of harmane.

CONCLUSIONS

Room temperature phosphorescence of harmane is reported for the first time and a SSRTP method for its determination was developed. Intense RTP was achieved in cellulose substrates containing either $AgNO_3$ or $TlNO_3$ as phosphorescence enhancers. Optimized experimental and instrumental conditions allowed

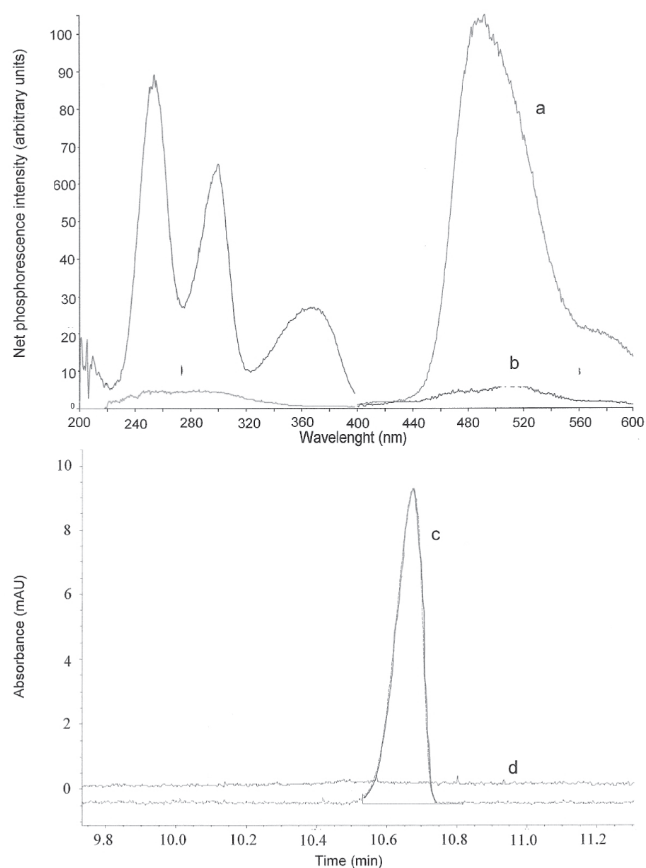

Figure 5. *(A) $TlNO_3$ enhanced room-temperature phosphorescence spectrum for a mixture of harmane and thalidomide (a) and for thalidomide (b); (B) MECK electropherogram for a mixture of harmane and thalidomide (c) and for thalidomide (d)*

excellent sensitivity and satisfactory dynamic linear range to be achieved. Interference studies were made and the experimental results indicated a selective capability of the method towards several concomitant substances (two indolic alkaloids, thalidomide and zidovudine). A simulated harmane tablet, containing thalidomide as a concomitant drug, was successfully analyzed by the method. When compared to the results achieved using MECK, it has been demonstrated that SSRTP can be potentially used for such analytical application. SSRTP method is simple and selective, eliminating the need for neither powerful separation methods (HPLC[20], for instance) to isolate the analyte from the tested potentially interferent substances nor multivariate approaches[24] for the determination of harmane in mixtures.

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