Room-temperature Phosphorimetry for the Selective Determination of Yohimbine in the Presence of Reserpine-like Indolic Alkaloids

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For centuries, extracts of the *Rawolfia* plants have been used to treat several diseases. It is now understood that the pharmacological properties of these plants come mainly from indolic alkaloids, which have been extensively used as active ingredients in pharmaceutical preparations.¹ Two major classes of alkaloids can be identified in such plants, the reserpine-like and the yohimbine-like indolic alkaloids.² Reserpine and rescinnamine are two of the principal components of the reserpine class, and are known to have potent tranquilizer, sedative and anti-hypertensive actions.³ Yohimbine and its stereoisomer, corynanthine, have important selective α -adrenergic blocker properties, and have been employed for the treatment of male impotency and arteriosclerosis. Despite these beneficial effects, non-desirable collateral effects due to continuous use of these compounds may arise.³ Analytical methodologies for the determination of such indolic alkaloids (especially yohimbine) in plant extracts (teas) or in associated commercial formulations are desirable. Because these two classes of indolic alkaloids present similarities in terms of molecular structure, a selective analytical methodology for the determination of yohimbine in the presence of the other indolic alkaloids without pre-separation procedures is very difficult.

While spectrophotometry^{4,5} presents poor sensitivity for indolic alkaloids (limits of detection, LOD, in the $\mu\text{g g}^{-1}$ range), better LOD (ng g^{-1} range) is achieved with fluorometry.⁶⁻⁸ However, fluorometric methods must be coupled with separation techniques, such HPLC,^{6,7} in order to allow a selective determination of indolic alkaloids. Voltammetry has shown good potential for the selective determination of reserpine in the presence of rescinnamine.⁹ However, no methodology has been optimized for the determination of yohimbine.

The potential of phosphorimetry for the sensitive determination of indolic alkaloids has been demonstrated (absolute LOD in the sub-ng range).¹⁰⁻¹³ Phosphorimetry has been established as a powerful technique for the trace determination of organic molecules,¹⁴ and its selectivity can be enhanced by optimizing several experimental variables.

In this work, a room-temperature phosphorescence (RTP) based analytical method was developed for the selective determination of yohimbine in the presence of reserpine-like indolic alkaloids (reserpine and rescinnamine) without previous separation procedures and using simple equipment. In a first

part, the RTP experimental conditions already reported¹¹⁻¹³ were tried, and only partial success was achieved. In a second part, new experimental conditions were evaluated in order to induce the RTP signal of yohimbine and to eliminate signals from the interferents. A UV treatment of acidic sample solutions allowed for the determination of yohimbine in binary mixtures containing either reserpine or rescinnamine at concentrations of up to 100-times higher.

Experimental

Apparatus

An Aminco Bowman spectrofluorimeter (SLM Instruments, Urbana, IL, USA) was employed. This instrument composed a 150 W xenon arc lamp (Conrad-Hanovia), two monochromators with 600 grooves/mm diffraction grades, a 1P21 photomultiplier tube (Hamamatsu Corp., Middlesex, NJ, USA), an SLM Aminco microphotometer and a Hewlett-Packard X-Y recorder (Model 7010 B, Palo Alto, CA). A rotating can-type phosphoroscope (Aminco-Keirs) was employed to collect phosphorescence without interferences from scattering or possible fluorescence. A laboratory sample holder was used.

A photochemical treatment of samples and paper background reduction were carried out in a Rayonet photochemical reactor loaded with seven lamps with maximum emission wavelengths at 250 nm and 300 nm.

Chemicals

Chromatographic paper (Whatman No. 1) treated, in the laboratory, for background reduction was employed as a sample substrate. The water used throughout this work was distilled in a glass apparatus. N_2 (White-Martins, Brazil) was used to bathe the samples in the holder. Ethanol was purchased from Merck (Brazil). Reserpine, rescinnamine, and yohimbine were provided by Sigma (USA). Potassium iodide (KI) and sodium dodecyl sulfate (SDS) were from BDH. Thallium nitrate (TlNO_3) was from Fluka. A reserpine-based pharmaceutical formulation (Reserpina Gross[®]) contaminated in the laboratory with yohimbine was used to test the method.

Procedures

Cellulose paper strips were washed out with water in a Soxhlet apparatus for 8 h and then irradiated in a UV reactor for an additional 8 h¹¹ for background reduction. Ethanol-water (50/50, v/v) was employed to prepare most of the analyte

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Table 1 Room-temperature phosphorescence signal of yohimbine^a in mixtures containing reserpine or rescinnamine in basic pH^b and in the natural pH of an ethanol-water solution^c

Mixture	Molar ratio	I_{YR}/I_Y^d			
		Natural pH	Natural pH	Natural pH	Basic pH
		(284/450) ^e	(274/444) ^e	(275/444) ^e	(312/460) ^e
Yohimbine:	1:1	1.12	0.96	1.02	1.07
Reserpine	1:10	2.31	0.49	0.99	1.04
	1:100	16.54	9.76	0.11	0.70
Yohimbine:	1:1	1.09	0.44	0.24	1.14
Rescinnamine	1:10	1.87	0.09	0.69	0.77
	1:100	7.52	19.51	0.10	8.32

a. Yohimbine 1×10^{-5} M. b. NaOH 1 M in ethanol-water (50/50, v/v). c. Ethanol-water (50/50, v/v). d. I_{YR}/I_Y is the ratio between the yohimbine signal in a solution containing a reserpine-like indolic alkaloid and the yohimbine signal. e. Excitation (λ_{exc}) and emission (λ_{em}) wavelengths used for measurements.

solutions. The pH of the solutions was varied by replacing the water for basic (NaOH) and acidic (HCl) aqueous solutions. Deionized water was used to prepare KI, $TiNO_3$ and SDS solutions.

RTP measurements

Five microliters of analyte solutions were deposited on the paper substrate with a micropipette. In some cases, the substrate was previously spotted with 5 μ L of a heavy atom solution or/and 5 μ L of a surfactant solution. Then, the substrates with the samples were dried in a vacuum oven at room temperature for 1 h. A desiccator was used to place the samples until just before the RTP measurements. Five minutes, prior to and during, the RTP measurement, a dry N_2 flow was passed through the substrate surface to minimize the quenching effects from O_2 .

Results and Discussion

Selective RTP determination of yohimbine in basic and natural pH

RTP of reserpine, rescinnamine and yohimbine in basic pH as well in the natural pH of the (50/50, v/v) ethanol-water solution in the presence of heavy atom enhancers (HAE's) on paper substrate were studied. Only I^- and Tl^+ (in the presence of SDS) were used as HAE's, since they are the most efficient for these indolic alkaloids.¹¹⁻¹³ Four different experimental conditions could be identified for the potential-selective determination of yohimbine in the presence of reserpine and rescinnamine. The experimental conditions selected were those that enabled a significant I_A/I_B (signal-to-blank ratio) for yohimbine (analyte) as well as small RTP signals for reserpine and rescinnamine (interferents). The differences in the maximum wavelengths of the excitation and emission ($\lambda_{exc}/\lambda_{em}$) between the analyte and interferents also improve the selectivity. Selective determinations using the selected experimental conditions were evaluated by comparing the RTP signals of a 10^{-5} M yohimbine standard solution (I_Y) with signals from solutions of yohimbine (10^{-5} M) containing different proportions of either reserpine or rescinnamine (I_{YR}). The results are displayed in Table 1. Three yohimbine-interferent molar ratios were tested, and the excitation and emission wavelengths

Table 2 Room-temperature phosphorescence of the indolic alkaloids^a with an acidic pH^b

Compound	$I_A/I_B^c; (\lambda_{exc}/\lambda_{em})^d$		
	—	KI 1 M	$TiNO_3$ 0.1 M SDS 0.07 M
Yohimbine	1.30; (302/482)	19.51; (280/440)	0.76; (287/474)
Reserpine	1.12; (286/490)	1.09; (275/475)	0.85; (281/485)
Rescinnamine	1.05; (298/482)	0.91; (225/515)	1.14; (281/474)

a. Concentration of analytes were fixed to 10^{-4} M. b. HCl 0.1 M in ethanol-water (50/50, v/v). c. I_A/I_B is the analyte signal-background ratio. d. λ_{exc} and λ_{em} are the RTP maximum wavelengths of excitation and emission, respectively.

used for the measurement were fixed at the optimized maximum for yohimbine. From the results, it was observed that the selective determination of yohimbine can be successfully performed (I_{YR}/I_Y close to unity) for samples containing equivalent concentrations of reserpine no matter which experimental condition was used. For samples containing rescinnamine, only the use of natural pH or basic pH in the presence of Tl^+ /SDS allowed a selective determination. In solutions where the reserpine concentration was increased by ten times, only two experimental conditions (basic pH in the presence of Tl^+ /SDS and natural pH in the presence of I^-) could be successfully used. No experimental condition allowed the selective determination of yohimbine in solutions containing rescinnamine 10-times more concentrated. At higher molar ratios (100-times more concentrated), severe interferences, which were caused by the presence of reserpine and rescinnamine, were found.

Since only a partial success was achieved using already known experimental conditions, a study was performed in order to find new potential experimental parameters that would allow a selective determination.

RTP characteristics of yohimbine, reserpine and rescinnamine using acidic pH and HAE

RTP signals of the three indolic alkaloids were measured in an acidic medium (HCl 0.1 M). The influence of HAE (Tl^+ and I^-) was also tested (Table 2). In the absence of HAE as well as in the presence of Tl^+ , no significant phosphorescence was observed for any of the indolic alkaloids. In contrast, a significant yohimbine signal was achieved when I^- was present. Under these experimental conditions, reserpine and rescinnamine (both at the 10^{-4} M concentration level) displayed weak phosphorescence, indicating this experimental condition as a potential candidate for selective determination.

RTP characteristics of yohimbine, reserpine and rescinnamine after a treatment with UV radiation

A modification of the luminescence characteristics of organic compounds by a UV treatment of samples has been used to develop fluorometric¹⁵ and phosphorimetric^{13,16} analytical methods. UV radiation is employed to produce species with a higher luminescent quantum yield that would enable improvements in the sensitivity. Alternatively, this procedure can generate non-luminescent derivatives from previous luminescent compounds. This could have interesting consequences for the analysis of mixtures, since signals from concomitant species in the matrix containing the analyte of interest can be suppressed. Solutions of the three indolic alkaloids were treated with UV radiation in a photochemical

Table 3 Room-temperature phosphorescence of the indolic alkaloids^a at different pH's after a 60 min UV treatment

pH		I_A/I_B^c ; ($\lambda_{exc}/\lambda_{em}$) ^d		
		Yohimbine	Reserpine	Rescinamine
No HAE	Natural ^d	2.50; (310/498)	1.85; (292/469)	1.98; (289/465)
	Basic ^e	17.45; (320/460)	12.85; (376/440)	13.68; (376/438)
	Acid ^f	51.48; (306/496)	0.92; (322/488)	0.98; 320/488
KI	Natural	4.04; (290/488)	3.02; (312/458)	2.84; (310/462)
	Basic	26.44; (278/448)	5.55; (305/466)	7.73; (312/466)
	Acid	6.05; (350/500)	1.06; (279/440)	0.95; (277/438)
TiNO ₃ /SDS	Natural	6.02 (288/485)	1.63; (308/462)	1.48; (306/460)
	Basic	14.55; (324/492)	6.83; (375/438)	11.54; (376/438)
	Acid	18.61; (304/496)	0.92; (284/442)	1.02; (280/445)

a. Concentration of analytes were fixed to 10^{-4} M. b. I_A/I_B is the analyte signal-background ratio. c. λ_{exc} and λ_{em} are the RTP maximum wavelengths of excitation and emission, respectively. d. Ethanol-water (50/50, v/v). e. NaOH 1 M in ethanol-water (50/50, v/v). f. HCl 0.1 M in ethanol-water (50/50, v/v).

reactor, and then deposited in a paper substrate pretreated or not with HAE's. For this experiment, a 60 min irradiation time was selected. The results are displayed in Table 3.

In a basic medium, irradiated yohimbine displays RTP signals in both the absence and presence of HAE's. Irradiated rescinamine and reserpine also had significant RTP signals under such conditions, and their magnitude and spectral locations make the selective determination of yohimbine using UV irradiated samples in basic medium impossible.

At the pH of the ethanol-water solution, irradiated samples of all three alkaloids presented weak signals. RTP signals for the three indolic alkaloids were observed when I^- was present in the substrate, but $\lambda_{exc}/\lambda_{em}$ was too close, thus making a selective determination not viable. The results in the presence of Ti^{+} were not significant.

The most significant result concerning the selective determination of yohimbine was achieved in an acidic pH, where a strong signal for irradiated yohimbine was obtained (I_A/I_B of around 50 at 306/460 nm). The presence of Ti^{+} /SDS or I^- induced weaker signals. Since RTP of reserpine and rescinamine were not significant in UV-treated acidic solutions, irradiated samples at acidic pH in the absence of HAE is an experimental condition that appears to be suitable for the selective determination of yohimbine.

Maximization of the yohimbine RTP signal

From the studies mentioned above, two new experimental conditions were found to have potential for developing a selective determination method for yohimbine. The first methodology is based on a UV treatment of the sample using acidic pH; the second one uses non-irradiated acidic analyte solutions and I^- as the HAE. In order to improve the sensitivity, the experimental parameters of these two methodologies were optimized. For the methodology based on the UV treatment, it was necessary to optimize the concentration of HCl and SDS as well as the UV exposure time. For the second methodology, the concentration of HCl in the analyte solution and the concentrations of KI and SDS solutions to be deposited on the substrate were optimized.

Irradiation time. The effect of the UV radiation exposure time 30 to 90 min on the yohimbine RTP signal was studied. The concentration of HCl was fixed at 0.1 M and the volume of ethanol in the solvent system was 20%. A signal dependence on the exposure time was found. From 30 to 45 min of exposure, a

sharp increase of the RTP signal was observed. The maximum signal intensity (I_A/I_B around 60) was achieved within 45 min. With longer exposure times, a decreased signal intensity occurred. A 45 min UV irradiation time was used in further experiments.

Concentration of HCl. Concentrations of 0.05 – 1 M HCl were tested. It was found that for UV-irradiated yohimbine, the maximum RTP signal was obtained with HCl above 0.5 M. In the case of non-irradiated analyte solutions in the presence of I^- , the maximum RTP signal decreased for solutions with HCl above 0.1 M. For further experiments, HCl concentrations were fixed at 0.5 and 0.1 M for irradiated and non-irradiated yohimbine samples, respectively.

Proportion of ethanol in the solution. Ethanol proportions from 20 to 80% in the analyte solution were tested. For irradiated acidic solutions, the maximum RTP signal was observed up to 30% of ethanol; for non-irradiated yohimbine solutions, the RTP intensity in an I^- -treated substrate dropped when ethanol exceeded 50%.

Concentration of SDS and HAE. For non-irradiated yohimbine, the concentration of the KI solution deposited on the substrate was optimized. It was found that HAE concentrations above 0.5 M were sufficient to induce the maximum RTP signal. The influence of SDS on the substrate was also tested. No significant signal improvement was observed for non-irradiated analyte solutions. In the case of UV-treated yohimbine, the presence of SDS decreased the signal.

Selective determination of yohimbine using methodologies involving an acidic pH

Two optimized RTP methodologies based on an acidic pH were tested in order to verify the possibility for the selective determination of yohimbine in the presence of reserpine or rescinamine. This was done by comparing the RTP signal of a 10^{-5} M yohimbine standard solution with RTP signals from solutions of yohimbine containing different proportions (10^{-5} , 10^{-4} and 10^{-3} M) of either reserpine or rescinamine. The results are displayed in Table 4. For the methodology without a UV treatment, and in the presence of I^- , a selective determination was possible only for mixtures containing interferent-analyte molar ratios of 1 and 10. For mixtures containing concentrations of the interferents 100-times higher than the analyte, a strong spectral interference was observed. A better result was achieved for UV-irradiated samples. In this case,

Table 4 Room-temperature phosphorescence signal of yohimbine^a in mixtures containing reserpine or rescinnamine with an acidic pH^b

Mixture	Molar ratio	I_{YR}/I_Y^c	
		UV treated (306/496) ^d	KI 1 M (280/440) ^d
Yohimbine:Reserpine	1:1	0.95	1.05
	1:10	1.04	1.02
	1:100	1.13	1.36
Yohimbine:Rescinnamine	1:1	1.01	0.98
	1:10	1.04	1.02
	1:100	1.16	1.45

a. Yohimbine 1×10^{-5} M. b. HCl 0.5 M. in ethanol-water (50/50, v/v). c. I_{YR}/I_Y is the ratio between the yohimbine signal in a solution containing a reserpine-like indolic alkaloid and the yohimbine signal. d. Excitation and emission ($\lambda_{exc}/\lambda_{em}$) wavelengths used for measurements.

satisfactory results for the selective determination of yohimbine were achieved in the presence of reserpine-like indolic alkaloids, even in mixtures with interferent-analyte molar ratios of 100.

Analytical figures of merit

Analytical curves for the RTP method based on the UV treatment of acidic solutions were constructed by using yohimbine standard solutions. The LOD (based on $3s_b$, where s_b is the standard deviation of 16 blank measurements) was 2×10^{-7} M and the relative standard deviation (RSD, $n = 16$) at the 10^{-5} M level was 9.6%. The linear dynamic range (LDR, $r = 0.989$) was limited to only two orders of magnitude. This LDR is smaller than that reported for the yohimbine RTP methods where no UV treatment is employed.¹² This result indicates that the kinetics of the photochemical reaction involves an additional component that affects the linear relationship between the analyte concentration and the signal. However, if the analyte concentration is carefully adjusted into the linear part of the analytical curve, the method can be successfully applied.

Determination of yohimbine in a reserpine-based drug

A method was tested with two different pharmaceutical tablets. The first one was a commercial reserpine-based pharmaceutical formulation (0.25 mg per tablet) which has reserpine as the only active ingredient. Twelve tablets were pulverized with a pestle in a porcelain mortar and weighed. Then, a small amount of yohimbine (0.3 mg) was added to the pulverized powder and mixed for 1 h in a mechanical shaker. The powder was washed with ethanol-water (30/70 v/v) to dissolve the indolic alkaloids. The solution was vacuum filtered and then diluted with an acidic pH solution. The final sample solution had approximately a 10-to-1 reserpine-yohimbine molar proportion. The second formulation was made in the laboratory by mixing 75 mg of reserpine and 1 mg of yohimbine with 5 g of excipient material (analytical-grade starch). The powder was mixed in a mechanical shaker for 1 h. Portions of the mixture (0.5 g) were pressed in a pellet press. Each 0.5 g tablet had approximately 7.5 mg of reserpine and 0.1 mg of yohimbine. Four of these tablets were weighed, pulverized and washed out with ethanol-water (30/70 v/v). The solution was vacuum filtered and diluted to the appropriate volume with an acidic solution. The final sample solution had approximately a 50-to-1 reserpine-yohimbine molar proportion.

Aliquots of the samples were UV irradiated for 45 min before the analysis. The recoveries achieved using analytical curves were $96.1 \pm 8\%$ for the spiked commercial formulation and $107.2 \pm 11\%$ for laboratory-made tablets. These results were based on six determinations.

Conclusion

This paper demonstrates the possibility for the RTP-selective determination of yohimbine in the presence of reserpine-like indolic alkaloids using simple instrumentation. Among several experimental conditions tested, the UV treatment of acidic samples has enabled a selective yohimbine determination in samples containing a concentration of concomitant alkaloids as high as 100 times. The LOD and RSD are comparable to other RTP methods for indolic alkaloids.¹¹⁻¹³ LDR was limited to about two orders of magnitude, therefore, a careful adjustment of the analyte concentration in the sample is necessary when the analysis is performed. The method was tested in reserpine-based pharmaceutical formulations spiked with yohimbine. The recovery was found to be satisfactory.

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