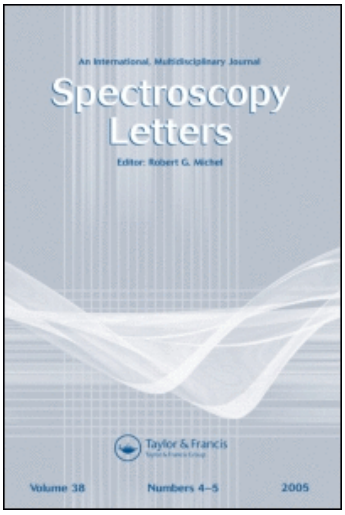

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Colorimetric and Spectrofluorimetric Methods for the Determination of Melatonin in Tablets and Serum

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COLORIMETRIC AND SPECTROFLUORIMETRIC METHODS FOR THE DETERMINATION OF MELATONIN IN TABLETS AND SERUM

Keywords Colorimetry; modified van Urk reaction; spectrofluorimetry; ortho-phthalaldehyde; tablets; serum

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ABSTRACT:

Two sensitive and accurate colorimetric and spectrofluorimetric methods are presented for the determination of melatonin in tablets and serum. The first method utilizes the reactions of p-dimethylaminobenzaldehyde in hydrochloric acid (van Urk reagent)-ferric chloride in sulphuric acid (Salkowski reagent)

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mixture. The blue color of the resulting reaction product is measured at 630 nm. The second method is based on the reaction of melatonin with o-phthalaldehyde in acid medium which yields highly fluorescent condensation product that is measured at 465 nm as emission wavelength, using excitation wavelength at 355 nm. No interference was observed from tableting additives, and the applicability of the methods was examined by analysing tablets containing melatonin (single and combined with pyridoxine). Mean percentage recoveries from tablets were found to be 99.9±0.31 for single and 100.5±0.15 for combined tablets using colorimetric method, while by applying spectrofluorimetric method the recoveries were found to be 100.6±0.41 for single and 100.2±0.39 for combined tablets. Furthermore, the proposed methods were extended to the in-vitro determination of melatonin in serum. The detection limits are 0.27 ug ml^{-1} for colorimetric method and 0.00035 ug ml^{-1} for spectrofluorimetric method.

INTRODUCTION

Melatonin, N-acetyl-5-methoxytryptamine, is a hormone produced in the pineal gland. Results from animal studies, indicate that melatonin increases concentration of aminobutyric acid and serotonin in the midbrain and hypothalamus[1]. Melatonin is involved in inhibition of gonadal development, control of oestrus, and in protective changes in skin coloration. In addition melatonin synchronizes circadian and circannual rhythms, stimulates immune function, and inhibits cancer progression and promotion[2].

Screening of the literature revealed that only few analytical techniques have been developed for the determination of melatonin in human urine, plasma, rat pineal and aqueous solutions, these include, high performance liquid chromatography with fluorescence detection[3-5] or high performance liquid chromatography with electrochemical detection[6], gas chromatography[7,8] and radioimunoassay[9]. Recently, we have reported a derivative spectrophotometric method and a spectrofluorimetric procedure (based on the intrinsic fluorescence) for determination of melatonin-pyridoxine combination[10].

The chromogenic reagent "van Urk-Salkowski reagent (VUS) consisting of p-dimethyl aminobenzaldehyde (p-DMAB) in concentrated hydrochloric acid-ethanol (van Urk reagent) and ferric chloride ($FeCl_3$) in concentrated sulphuric acid-water (Salkowski reagent) was introduced as a sensitive reagent for thin-layer chromatographic detection and identification of indole derivatives[11]. Melatonin, being indole derivative, was reported to produce an extremely stable blue chromophore on the thin-layer plates after spraying with this chromogenic reagent[11].

The fluorescence reaction of o-phthalaldehyde (OPA) as a sensitive test for indole derivatives was described[12]. This reagent was subsequently used for the quantitative estimation of 5-hydroxytryptamine in rat brain[13] and in human urine[14] . Also, OPA was used as a spray reagent for detection of imidazoles and indoles[15], and was applied as a spray reagent for identification and quantification of some indole derivatives on thin-layer chromatograms[16].

The aim of this work was to apply the VUS and OPA reagents, respectively, for colorimetric and spectrofluorimetric assay of melatonin in bulk form and in pharmaceutical formulations. Method A describes the colorimetric determination of the blue colored condensation product formed between melatonin and VUS reagent at 630 nm. In method B, melatonin reacts with OPA to give a highely fluorescent product with excitation and emission wavelengths at 355, 465 nm, respectively.

The above described methods were successfuly applied for the determination of melatonin in serum. Analytical quality criteria, including linearity, sensitivity, precision, accuracy and recovery are discussed.

EXPERIMENTAL

Apparatus

Colorimetric determination were performed using a Perkin-Elmer Model 550S UV-visible spectrophotometer and a Hitachi Model 561 recorder.

All fluorimetric measurements were performed on a Perkin-Elmer Model 650-10S spectrofluorimeter equipped with 1 cm quartz cuvettes, a 150-W xenon lamp, excitation and emission grating monochromators, and a Perkin-Elmer Model 56 recorder.

Materials and reagents

All experiments were performed with analytical grade chemicals and solvents.

(A) van Urk reagent[11]: 1 g p-DMAB (Aldrich) was dissolved in 50 ml concentrated hydrochloric acid (s.g. 1.19) and 50 ml ethanol was added, this

reagent is stable for several months at room temperature when stored in a brown glass bottle.

(B) Salkowaski reagent[11]: 0.203 g ferric chloride ($FeCl_3$. $6H_2O$) was dissolved in 50 ml water and 30 ml concentrated sulphuric acid (s.g. 1.84).

(C) VUS reagent: the working reagent used was made up of reagent A and B (1:4), this reagent may be kept at room temperature for several weeks.

(D) OPA reagent (sigma, U.S.A.), 0.05 % w/v was prepared by dissolving 50 mg OPA in 100 ml absolute methanol.

Authentic samples of melatonin were kindly donated by Pharco Pharmaceuticals, Alexandria, Egypt and were used without further purification. Melatonin(R) tablets (Pharco Pharmaceuticals, Alexandria, Egypt) labelled to contain 5 mg melatonin, and Viva-Max 3(R) tablets (Amoun Pharmaceutical. Kahira, Egypt) labelled to contain 3 mg melatonin and 10 mg pyridoxine hydrochloride were obtained from the market.

Serum specimen were collected from adult healthy volunteers who were not under medical treatment and stored frozen at-20°C.

Standard solution and calibration graph for Method A:

Stock standard solution of melatonin (0.125 mg ml^{-1}) were prepared in ethanol and stored refrigerated at 4°C in brown glass flasks. Aliquots from stock standard solution (0.125 mg ml^{-1}) ranging from 0.1 to 0.8 ml (0.1 ml steps) was transferred into 10-ml volumetric flasks. To each flask, 2-ml of VUS reagent was added and the flasks were stoppered and heated in a water-

bath at 60°C for 20 min. Then, the flasks were cooled to room temperature and diluted to volume with distilled water. The absorbance was measured against a reagent blank at 630 nm using 1-cm cells.

Standard solution and calibration graph for Method B:

A working standard melatonin solution (0.002 mg ml^{-1}) was prepared by appropriate dilution of stock standard melatonin solution with ethanol. Aliquots of the working standard solution (0.002 mg ml^{-1}) ranging from 0.1-0.4 ml (0.1 ml steps) were transferred into 10-ml volumetric flasks. To each flask, 0.1 ml OPA reagent and 1 ml 5M hydrochloric acid solution were added, successively. The flasks were stoppered and heated in a water-bath at 80°C for 10 min., then cooled and completed to volume with ethanol. The fluoresence intensities at 465 nm emission with excitation at 355 nm were measured. The observed fluorescence was corrected by subtracting the fluorescence intensity measured using reagent blank.

Procedure for the determination of melatonin in tablets:

Twenty tablets were weighed and finely powdered. A portion of the mixed powder equivalent to about 12.5 mg melatonin was accurately weighed, transferred to a 100-ml volumetric flask containing about 50-ml ethanol and extracted by shaking for 5 min. and then diluted to volume with ethanol and filtered.

For Method A:

Aliquots of the filtrate within concentration range cited in Table 1 were treated as under preparation of calibration graph.

TABLE 1

Analytical Data of the Calibration Graphs for the Determination of Melatonin by the Proposed methods.

Method	Selected wavelength λ(nm)	Concentration range ug/ml	Linear regression			$S_{y/x}$ [+]	S_a [++]	S_b [+++]	RSD* (%)	Er %**	Apparent molar absorpitivity ($l\ mol^{-1}\ cm^{-1}$)
			Intercept (a)	Slope (b)	Corr. coff. (r)						
-Colorimetric	630	1.25-10	$8.195X10^{-1}$	0.090	0.9999	$4.36X10^{-3}$	$3.064X10^{-3}$	$6.11X10^{-4}$	1.11	0.07	$2.1X10^{4}$
-Spectro-fluorimetric	λ_{ex} 355 λ_{em} 465	0.02-0.08	2.005	681.5	0.9999	0.143	0.305	0.567	0.981	1.01	

\+ $S_{y/x}$ = standard deviation of residuals
++ S_a = standard deviation of intercept of regression line
+++ S_b = Standard deviation of slope of regression line
* Relative standard deviation (n = 5)
** Percentage relative error

For Method B:

An aliquot, of the filtrate equivalent to 0.20 mg melatonin was transferred to a 100-ml volumetric flask and diluted to volume with ethanol to give a working test solution. Aliquots of the latter within concentration range cited in Table 1 were treated as under preparation of calibration graph.

Procedure for the in-vitro determination of melatonin in serum:

Aliquots of stock standard solution of melatonin 0.125 mg ml^{-1} (for method A) and 0.002 mg ml^{-1} (for method B) within concentration range cited in Table 1 were transferred into 10-ml stoppered shaking tubes, each containing 2 ml of serum. The contents were mixed by shaking for 2 min., then centrifuged at 5000 rpm for 20 min. Volumes of the centrifugate (0.1-0.8 ml for method A and 0.1-0.4 ml for method B) were transferred to 10-ml volumetric flasks and treated as above for method A or B starting from addition of the reagents VUS or OPA, respectively.

RESULTS AND DISCUSSION

Optimisation of colorimetric and spectrofluorimetric reaction variables:

The optimum conditions for the development of methods A and B were established by varying the colorimetric (or spectrofluorimetric) reaction parameters one at a time, keeping the others fixed, and observing the effect produced on the absorbance (or fluorescence) reading.

Optimisation of method A variables:

The effect of varying the ratio of p-DMAB (van Urk reagent) to $FeCl_3$ (Salkowski reagent) to form the VUS reagent was studied. Fig. 1-a shows that

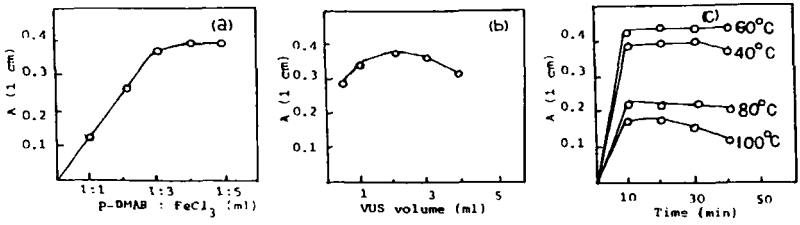


Fig. 1 : Effect of (a) p-DMAB : $FeCl_3$ ratio (b) volume of VUS (c) heating temperature and time, on the color intensity developed from 5 ug ml^{-1} melatonin.

the absorbance rose sharply with increasing the $FeCl_3$ concentration up to the ratio 1:3. p-DMAB: $FeCl_3$. Constant absorbances were obtained over the range (1:3) to (1:5) reagents ratio. Hence a ratio of 1:4 was selected. The effect of VUS concentration on color formation (Fig. 1-b) suggests that 2 ml of VUS reagent is optimal. Fig. 1-c shows that the optimum heating temperature and heating time for full color development are 60°C and 20 min., respectively.

The absorption spectrum of the blue chromogenic species produced by the suggested procedure is shown to have maximum absorbance at 630 nm, as shown in Fig. 2. The blue color was found to be stable for at least 24 hours.

Optimisation of method B variables:

The OPA reagent concentration, strength of the acid, heating temperature, reaction time and stabilization of fluorescent products have been optimised to reach high fluorescent intensity, high stability and low blank reading. The effect of OPA reagent concentration on the fluorescence

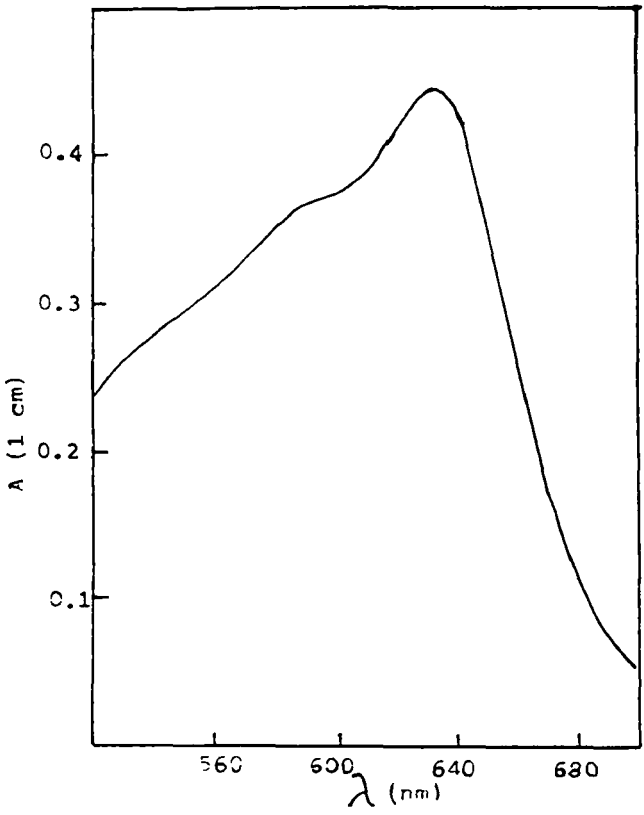


Fig. 2 : Absorption spectrum for the colored product of 5 ug ml^{-1} melatonin with VUS.

developed at the selected wavelength was attained by changing the concentration of OPA reagent over the range 0.025-0.10 % w/v. The maximum intensity was obtained using 0.05 % w/v (Fig. 3-a). Changing the molarity of hydrochloric acid over range 2-10 M showed that the optimum molarity of hydrochloric acid was 5 M. (Fig. 3-b). The effect of different heating tempera-

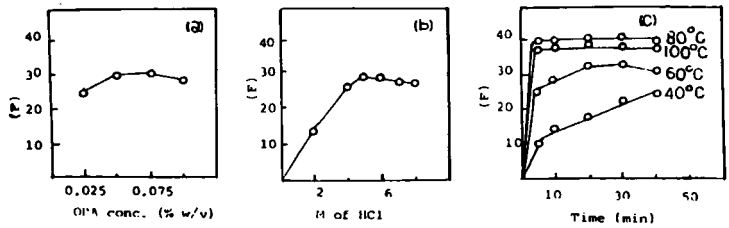


Fig. 3 : Effect of (a) OPA concentration (b) molarity of hydrochloric acid (c) heating temperature and time, on the fluoresence intensity developed from 0.06 ug ml^{-1} melatonin with OPA.

tures (40°, 60°, 80° and 100°C) for different times up to 45 min. was investigated. The higher fluorescence intensity was observed at 80°C after heating for 10 min. (Fig. 3-c). Using the above described experimental conditons the excitation and emission wavelengthes are 355 and 465 nm. respectively, (Fig. 4). The fluoresence intensity of the reaction product was found to be stable for at least 1 hour.

Chemistry of the color reaction:

Melatonin is an indole derivative having a free C-2 position and $-CH_2,CH_2NHCOCH_3$ side chain at the C-3 position [Scheme 1, (I)]. It has been reported that condensation of the indole derivative with p-DMAB (van Urk reaction) occurs at the free C-2 position and results in a violet-blue condensation product if C-3 position has a $-CH_2$-R group[17]. Modifying the typically van Urk reaction. the structure of the blue condensation product

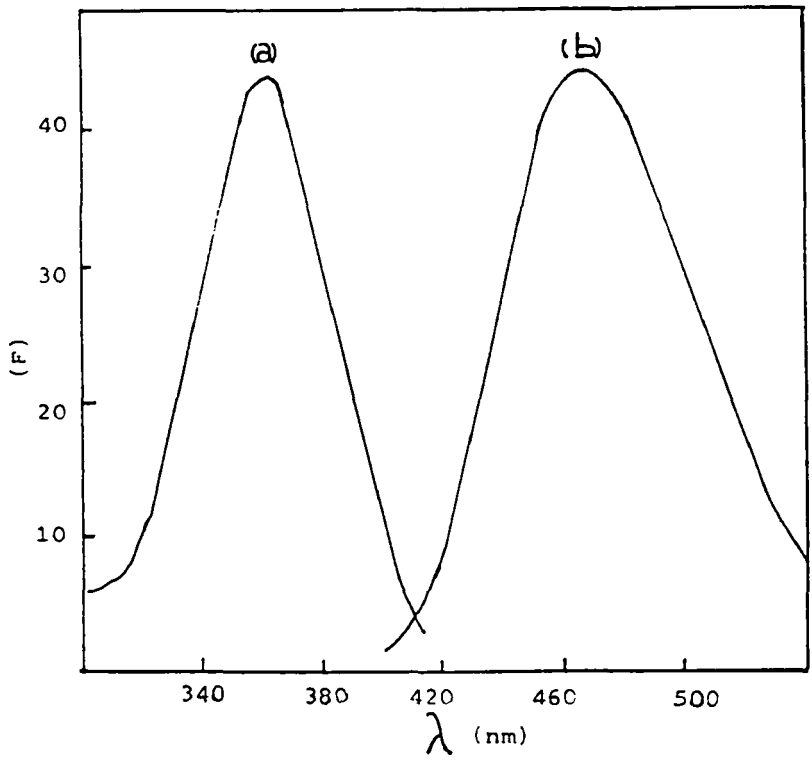
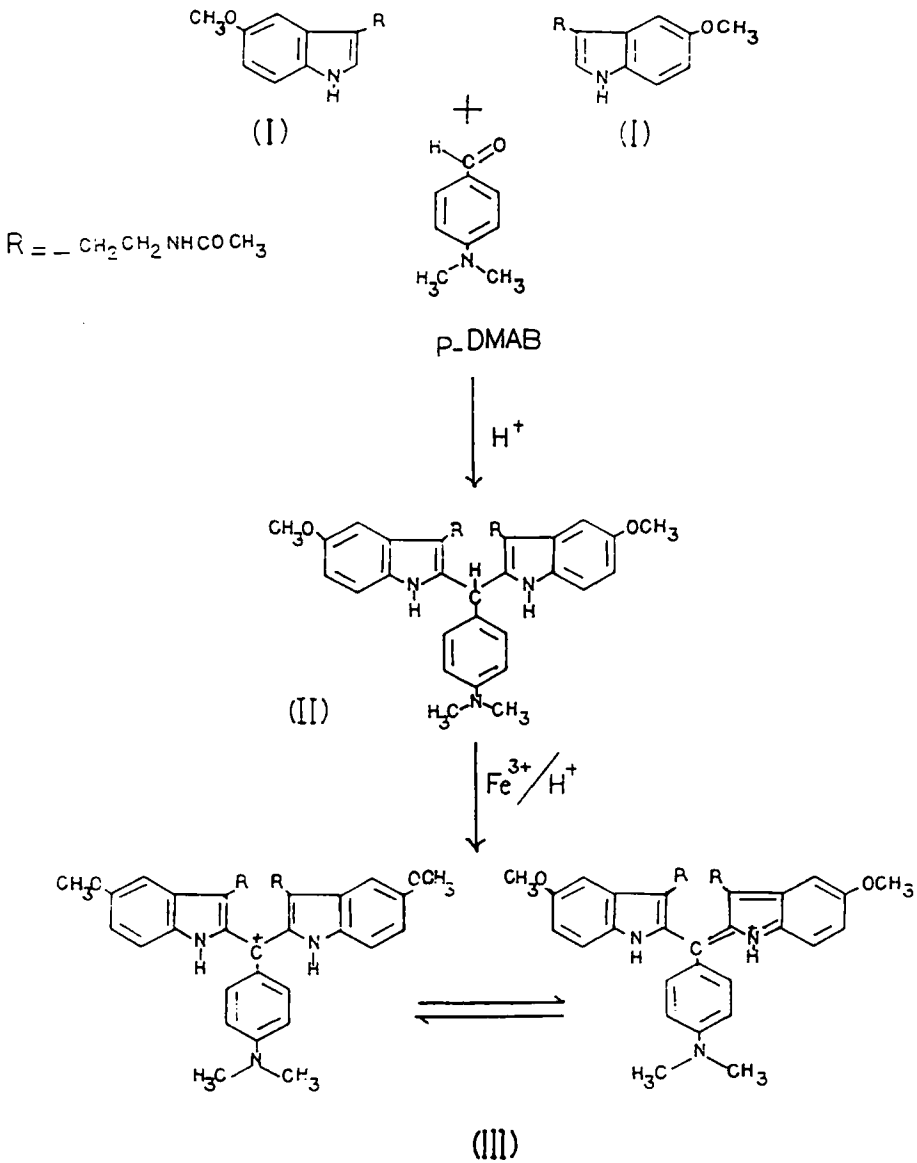


Fig. 4 : Excitation (a) and Emission (b) spectera for the fluorescent product of 0.06 ug ml^{-1} melatonin with OPA.

obtained in case of ergot alkaloids (3-substituted indole derivative having a free C-2 position) was illucidated and isolated[18].

Based on these findings, a proposed mechanism for the blue colored products formed by the reaction of melatonin with VUS reagent (p-DMAB/H^{+}/Fe^{+3}) is shown (Scheme 1). Condensation between melatonin (I) and p-DMAB in presence of HCl forms the diindolylphenylmethane derivative



SCHEME 1

(II) that is readily oxidised by Fe^{3+} ions to form the blue colored products (III). We have noticed that addition of p-DMAB/H^+ to melatonin (in absence of Fe^{3+}) produces the blue color but after a long time. Therefore, it is obvious that Fe^{3+} is playing a key role, as the oxidant, in formation of the blue colored product (III).

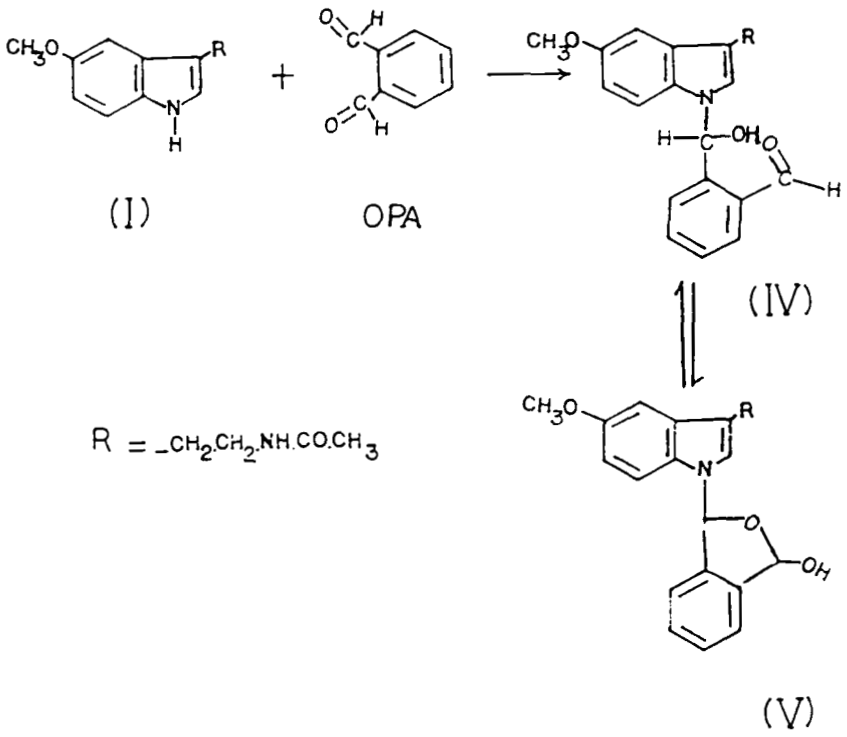
Chemistry of the fluorescence reaction:

The 3-substituted indoles were found to react with o-phthalaldehydic acid to form the 1-phthalidylindole derivative[19]. The latter is resulting from lactonization of the intermediate indolycarbinol. The fluorescent derivative obtained by reaction of OPA with melatonin (I) is proposed to be the indolylcarbinol (IV) that is resonating with the cyclic hemiacetal (V) resulting from intramolecular nucleophilic addition (Scheme 2).

Analytical data:

The calibration graphs were constructed for the two proposed methods (A & B) from five points over concentration range cited in Table 1. Regression analysis indicates linear relationships with negligible intercepts. Table 1 presents the results of the statistical analysis of the experimental data: regression equations calculated from calibration graphs, along with the standard deviation of the slope (S_b) and intercept (S_a) on the ordinate and the standard deviation of residuals ($S_{y/x}$). The high values of the correlation coefficients of regression equations indicate good linearity and conformity to Beer's law.

Five replicate determination at different concentration levels were carried out to test precision and accuracy of the proposed methods. The



SCHEME 2

relative standard deviations (RSD%) were found to be less than 2% indicating reasonable repetability of the methods (Table 1) and the relative standard errors (Er %) were found to be less than 1.01 % indicate the high accuracy of the proposed methods.

The detection limits[20] for melatonin using colorimetric method and spectrofluorimetric method were found to be 0.27, 0.00035 ug ml^{-1}, respectively, while the quantification limits[21] were found to be 0.88 and 0.0012 ug ml^{-1}, respectively.

Analysis of tablets:

The validity of the proposed methods for pharmaceutical preparations and the effect of possible interferences were studied by assaying Melatonin(R). tablets (labelled to contain 5 mg of melatonin per tablet) and Viva Max 3(R) tablets (labelled to contain 3 mg of melatonin and 10 mg pyridoxine hydrochloride). The results are accurate and precise, as indicated by the excellant % recovery and RSD % less than 0.41 and Er % less than 0.18 (Table 2). Application of the t- and F- tests showed that there was no significant difference in precision and accuracy between the colorimetric and the spectrofluorimetric methods.

In-vitro determination of melatonin in spiked human serum using the proposed methods.

The proposed methods were further applied to the determination of melatonin in spiked human serum without any interference from endogenous constituents in serum. Within-day precision (random analytical variation) was evaluated by replicate analysis of serum samples containing melatonin at concentration range cited in Table 3. Day-to-Day precision (total analytical variation) was similary evaluated on several days over two weeks and no more than one assay per day for each concentration. The data presented in Table 3 indicate that the 24 h and day-to-day coefficients of variation are generally lower than 0.52 %.

TABLE 2

Determination of Melatonin in Tablets by Colorimetric Method and Spectrofluorimetric Method.

Method	Colorimetric	Spectrofluorimetric
1- Melatonin(R) tablets,		
Mean*±S.D.	99.9 ± 0.31	100.6 ± 0.41
RSD(%)	0.31	0.41
Er(%)	0.139	0.183
t**	2.72	
F**	1.7	
2- Viva-Max 3(R) tablets		
Mean* ± S.D	100.5 ± 0.15	100.2 ± 0.39
RSD(%)	0.15	0.39
Er(%)	0.07	0.174
t**	1.39	
F**	6.76	

* Average of 5 determinations.

** Theoretical values of t and F- test at P = 0.05 are 2.31 and 6.93 respectively.

TABLE 3

Within-Day and Day-to-Day Precision and Relative Recovery in the Determination of Melatonin in Spiked Human Serum.

Method	Colorimetric			Spectrofluorimetric	
	% Recovery* (RSD. %)**			% Recovery* (RSD. %)**	
Add ug/ml	Within-Day	Day-to-Day	Add ug/ml	Within-Day	Day-to-Day
2.5	99.5(0.52)	99.9(0.41)	0.02	98.9(0.17)	99.4(0.09)
5	100.3(0.16)	100.7(0.12)	0.04	101.1(0.09)	100.7(0.08)
10	100.6(0.11)	100.3(0.08)	0.08	100.1(0.13)	100.7(0.11)

* Mean of five experiments.
** Relative standard deviation.

In conclusion, the proposed colorimetric and spectrofluorimetric procedures provide simple and sensitive methods suitable for the quality control analysis of melatonin in dosage forms and in biological fluids.

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