ACID–BASE AND TAUTOMERIC EQUILIBRIA OF HARMOL IN THE GROUND AND FIRST EXCITED SINGLET STATES

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Summary

Four molecular species have been identified from the acid–base equilibria of the alkaloid harmol by using absorption and fluorescence spectroscopy. The pK_a values which govern the acid–base equilibria in the ground electronic state have also been derived from the absorption spectra. The corresponding pK_a values for the first excited singlet state equilibria have been calculated by means of the Förster equation. Through auxiliary experiments in the dispersed solid phase, it has been possible to separate the emission maxima of the neutral and zwitterionic species in aqueous media. As occurs in harmalol, in the first excited singlet state the hydroxy group is more acidic and the ring nitrogen atom more basic than in the ground state.

1. Introduction

Harmol (1-methyl-9*H*-pyrido[3,4-*b*]indol-7-ol) (HOB) is an alkaloid of the natural harmala group of molecules whose physiological and pharmacological properties have been described previously [1 - 5]. As can be seen from Fig. 1(a) the acid–base equilibria for harmol give a variety of molecular species depending upon the pH of the solution, as a consequence of the existence of two centres able to show acid–base behaviour (the phenolic group and the pyridinic nitrogen) together with a pyrrolic group which is a third centre of extremely low acidity.

The two apparent pK_a values pK_{a_1} and pK_{a_2} in Fig. 1(a) have been derived from the UV–visible absorption spectra [6]. In ref. 6 the existence of a mixture of neutral HOB and zwitterionic $^-OBH^+$ was found for solutions with a pH value between 8 and 9, but the question of the identification of the maxima corresponding to each molecular species was left unanswered and no reference to the individual values for the simple equilibrium constants K_1, K_2, K_3 and K_4 in Fig. 1(a) was reported.

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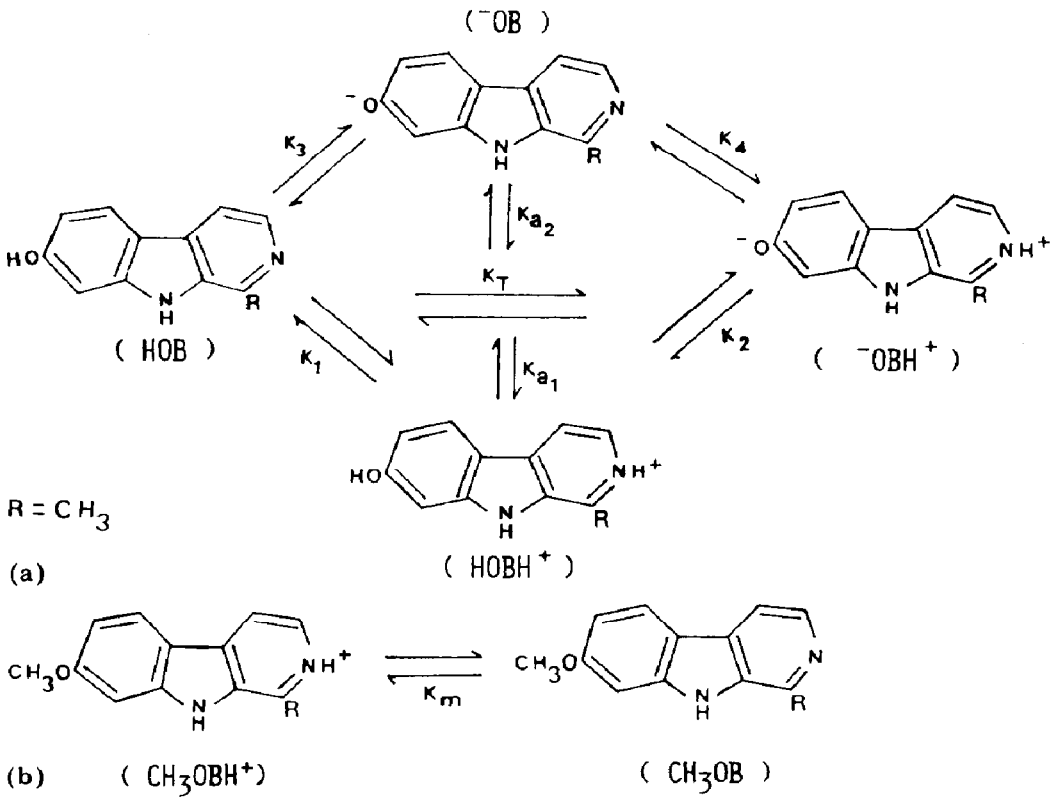

Fig. 1. Acid–base equilibria for (a) harmol and (b) harmine.

Several works concerning the fluorescence of harmol in organic solvents can be found in the literature [7 - 10] but the most recent revision of the acid–base equilibria for harmol in aqueous solutions has been made by Wolfbeis *et al.* [11]; taking into account the apparent pK_a values from the literature these authors have carried out a wide study of the acid–base equilibria in the first excited singlet state.

The work reported in this paper deals with the acid–base equilibria of harmol both in the ground and the first excited singlet states. As described below we have derived the pK_a values for the individual acid–base equilibria involving both the phenolic group and the pyridinic nitrogen in the ground state instead of the apparent global values reported in the literature. From these values found for the ground state and by means of the Förster equation [12] the pK_a values for the individual equilibria in the first excited singlet state have been revised.

Also, we have observed the emission of the neutral species HOB in aqueous solutions in the form of a fluorescence band of low intensity together with a band corresponding to the zwitterionic molecule $^-OBH^+$. The former was not referenced in the Wolfbeis work [11] where only the emission of the zwitterionic $^-OBH^+$ and acid $HOBH^+$ species were reported. The emission of the anionic ^-OB form has been also detected in the fluorescence

spectra in moderately basic solutions. No emission or absorption transitions corresponding to molecular species arising from a hypothetical acid–base equilibrium involving the pyrrolic N—H group (see Fig. 1(a)) have been found.

The fluorescence properties of harmol have proved to be more complex than those observed for the aromatic β-carbolines, *i.e.* norharman, harman and harmine [13], and show some similarity to those observed for harmalol [14] (the 4,9-dihydro derivative of harmol).

2. Experimental details

2.1. Materials

Harmol and harmol hydrochloride were obtained from the Sigma Chemical Company. Strongly acidic solutions were prepared with analytical grade hydrochloric acid and strongly basic solutions were prepared with analytical grade NaOH. For intermediate pH values the solutions were buffered with 0.1 M HAc–NaAc (Ac ≡ acetate) mixtures for the pH range 3 - 4, Na_2HPO_4–KH_2PO_4 mixtures (1/15 M) for pH values between 5 and 8.5 and 0.1 M glycine–NaOH mixtures for the pH range 9 - 13.

2.2. Apparatus

The UV absorption spectra were recorded using a Cary 219 spectrophotometer. Uncorrected fluorescence emission and excitation spectra were recorded using a Perkin–Elmer MPF-44A spectrofluorometer. The pH values were measured directly by means of a Radiometer 26 pH meter to within 0.01 of a pH unit. All the experiments were carried out on thermostatted solutions at a temperature of 25 ± 0.1 °C.

2.3. Determination of pK_a values

Provided it is assumed that on increasing the pH of the solution the two equilibria (1) and (2) proceed simultaneously, it can be shown that an equilibrium mixture of the two tautomeric species HOB and $^-OBH^+$ must exist whose equilibrium constant is K_T. Consequently, operating with harmol solutions, only the first and second apparent acid dissociation constants K_{a_1} and K_{a_2} can be derived from standard spectrophotometric methods [15] by means of the formulae

$$pK_{a_1}(S_0) = pH - \log \frac{A_{HOBH^+} - A}{A - A_{(HOB + {}^-OBH^+)}} \quad (1)$$

$$pK_{a_2}(S_0) = pH - \log \frac{A_{(HOB + {}^-OBH^+)} - A}{A - A_{-OB}} \quad (2)$$

A_{HOBH^+}, $A_{(HOB + {}^-OBH^+)}$, A_{-OB} and A are the absorbance values of a solution of pure $HOBH^+$, a solution of an equilibrium mixture of HOB and $^-OBH^+$, a solution of the pure anionic form ^-OB, and the solution under observation respectively, all with the same overall concentration of the alkaloid. The index S_0 denotes that the pK_a values belong to the ground state.

These apparent equilibrium constants are related to the individual equilibrium constants (see Fig. 1(a)) by the relations

$$K_{a_1} = K_1(K_T + 1) = \frac{K_2(K_T + 1)}{K_T} \tag{3}$$

$$K_{a_2} = \frac{K_3}{K_T + 1} = \frac{K_4 K_T}{K_T + 1} \tag{4}$$

In order to determine K_1, K_2, K_3, K_4 and K_T it is necessary to separate the two equilibria that arise from $HOBH^+$ species to give the tautomeric equilibrium mixture. To do this, we can employ the dissociation equilibrium of a related molecule where only one of these equilibria evolves, with the assumption that on going from our problem molecule HOB to the reference, *i.e.* harmine (7-methoxy-1-methyl-9*H*-pyrido[3,4-*b*]indole; CH_3OB), (see Fig. 1(b)) does not affect the basicity of the pyridinic nitrogen. Then, and following ref. 15, we can assume that the value of K_1 from Fig. 1(a) can be approximated by that of K_m from Fig. 1(b). Previously a value of 8.0 has been reported for the K_m of harmine [13]. As has been reported elsewhere [15], when the value of K_T lies within the range 0.01 - 100, the observed spectrum exhibits the bands of both tautomeric species, as has been observed for harmol.

The $pK_a(S_1)$ values for the first excited singlet state have been estimated from the Förster formula [12]

$$pK_a(S_1) - pK_a(S_0) = \frac{0.625}{T} \Delta\bar{\nu} \tag{5}$$

$\Delta\bar{\nu}$ (cm^{-1}) is the difference between the energies of the 0–0 electronic transitions in the "right" and "left" species of the corresponding equilibrium (see Fig. 1), and T is the absolute temperature. The energy of these 0–0 transitions has been determined by averaging the energies corresponding to the least energetic maximum of the absorption spectrum and the most energetic maximum of the fluorescence spectrum.

3. Results and discussion

3.1. Electronic absorption spectra

The electronic spectra of harmol in aqueous solutions in the pH range 1 - 13 are shown in Figs. 2(a) and 2(b).

The spectrum of harmol does not change significantly on going from pH 1 to pH 6; consequently, it can be concluded that for pH values less than 6 the predominant species is the acid form $HOBH^+$; the spectrum at pH 1 is the closest to that of $HOBH^+$, with the low intensity maximum at λ = 357 nm overlapped by the high intensity maximum at λ = 320 nm.

The spectrum of harmol is again invariable for solutions of pH greater than 12. The longest wavelength band in these basic solutions is well defined

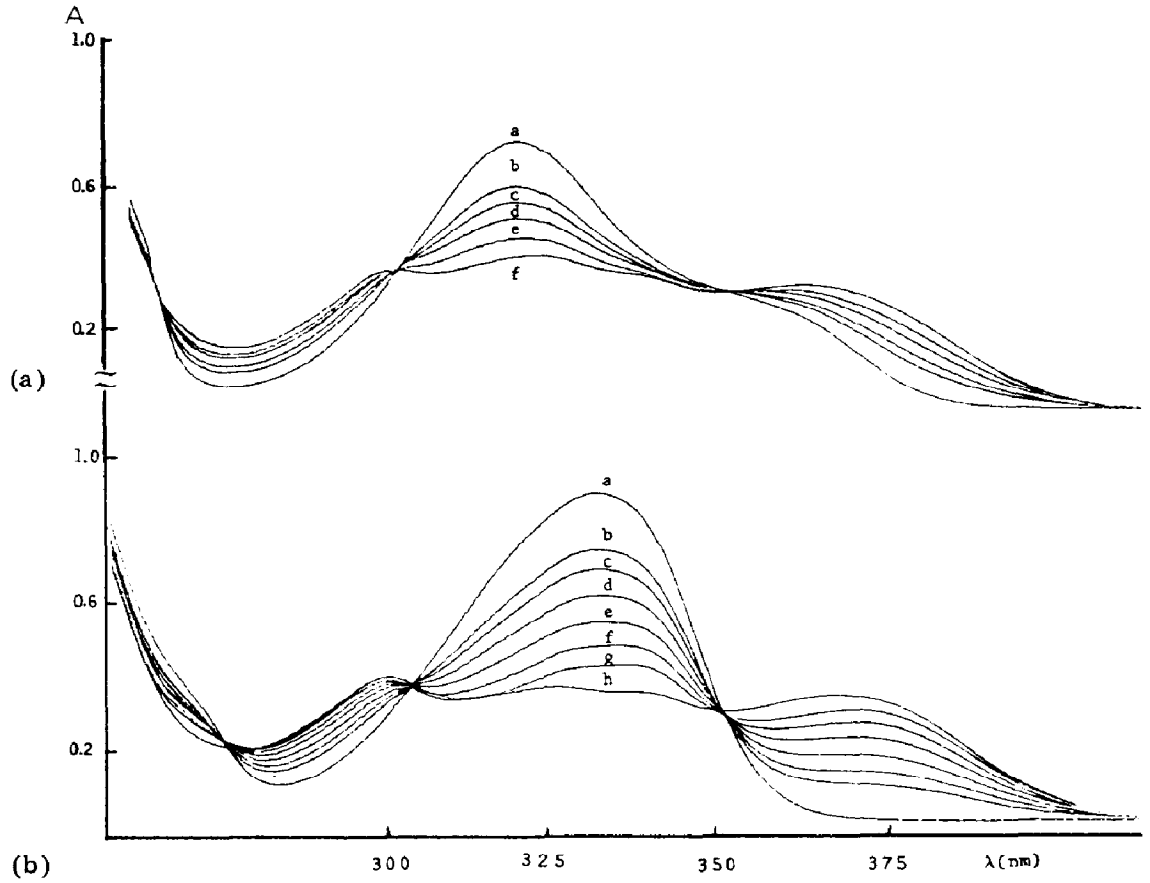

Fig. 2. Absorption spectra of harmol solutions (concentration $C = 5 \times 10^{-5}$ M; ionic strength $I = 0.1$): (a) pH 1.05 - 8.26 (curve a, pH 1.05; curve b, pH 7.47; curve c, 7.66; curve d, pH 7.88; curve e, pH 8.04; curve f, pH 8.26); (b) pH 8.43 - 13.00 (curve a, pH 13.00; curve b, pH, 10.03; curve c, pH 9.84; curve d, pH 9.63; curve e, pH 9.45; curve f, pH 9.25; curve g, pH 9.05; curve h, pH 8.43).

at $\lambda = 332$ nm, and in these solutions it can be considered that the dominant species is the basic form $^{-}$OB.

The characterization of the spectra of the individual tautomeric species is more difficult. There is no point between pH 6 and pH 12 where the spectrum remains constant. As can be seen below, the two apparent pK_a values are very similar and we can conclude that at pH values slightly higher than 6 a significant concentration of the acid form $HOBH^+$ must be present together with the two tautomeric forms HOB and $^{-}OBH^+$. Also, at pH values slightly less than 12, a significant concentration of the basic form $^{-}$OB must be present in solution.

The spectrum recorded at pH 8.4 gives the results most different from those recorded at pH 1 and pH 12. In this spectrum the absorbances at the wavelengths which correspond to the $HOBH^+$ and $^{-}$OB peaks are minima. Two new maxima are evident in the spectrum at pH 8.4 ($\lambda = 338$ nm and $\lambda = 367$ nm); in order to assign these maxima to either HOB or $^{-}OBH^+$,

some additional work has been done. The spectrum of neutral harmol HOB in tetrahydrofuran solution has been recorded (see Fig. 3), and no significant absorption has been observed beyond $\lambda = 340$ nm. Also, a strong band at $\lambda > 300$ nm is observed and much smaller bands are recorded at $\lambda = 321$ nm and $\lambda = 335$ nm. Consequently, the maximum at $\lambda = 367$ nm in aqueous solutions must be ascribed to the $^{-}OBH^{+}$ zwitterionic form, and that observed at $\lambda = 338$ nm must correspond to the HOB neutral form. This conclusion is reinforced by the behaviour observed for CH_3OB (a compound directly related to neutral HOB form); this also shows a maximum at $\lambda = 338$ nm and no significant absorption beyond $\lambda = 350$ nm.

All the related maxima are summarized in Table 1; the pK_a values are derived from eqns. (1) and (2). The absorbance values A_{-OB} and A_{HOBH^+} are

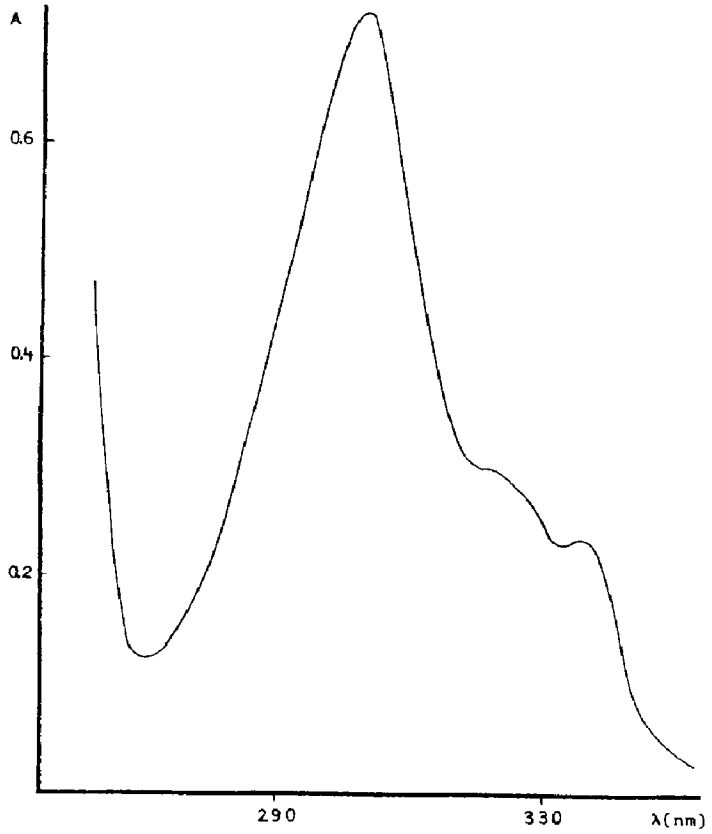

Fig. 3. Spectrum of neutral harmol in tetrahydrofuran solution.

TABLE 1

Wavelengths of the peaks of the absorption and fluorescence bands in harmol

	$\lambda_{max}(HOBH^+)$ (nm)	$\lambda_{max}(HOB)$ (nm)	$\lambda_{max}(^{-}OBH^{+})$ (nm)	$\lambda_{max}(^{-}OB)$ (nm)
Absorption	357	338	367	332
Emission	418	360	440	450

taken from the spectra recorded at pH 13 and pH 1.02 respectively. The value $A_{(HOB + {}^{-}OBH^{+})}$ must be found indirectly since at pH 8.4 significant quantities of both ^{-}OB and $HOBH^{+}$ together with the two tautomeric forms HOB and $^{-}OBH^{+}$ may be present owing to the similarity between pK_{a_1} and pK_{a_2}.

We have estimated $A_{(HOB + {}^{-}OBH^{+})}$ by using two solutions at moderately high pH values (9.63 and 9.84) where the presence of $HOBH^{+}$ can be disregarded but the concentration of ^{-}OB can be significant. By applying eqn. (2) to both solutions and assuming that pK_{a_2} does not change in this small pH range, the value $A_{(HOB + {}^{-}OBH^{+})} = 0.334$ was found.

All the absorbance measurements have been made at $\lambda = 330$ nm where all the species considered absorb significantly, and the values found are summarized in Table 2.

From the pK_a values found from eqns. (1) and (2), and taking into account the relations (3) and (4), we can derive the individual pK values for all of the equilibria outlined in Fig. 1(a). In this case the K_1 value is assumed to be the K_m value in Fig. 1(b). All the pK values are summarized in Table 3.

3.2. *Fluorescence spectra*

The fluorescence spectra of aqueous solutions of harmol over a wide pH range are depicted in Figs. 4(a) and 4(b). The excitation wavelength $\lambda = 303$

TABLE 2

Absorbance values of harmol at $\lambda = 330$ nm

pH	A
1.40	0.586 ($HOBH^{+}$)
7.47	0.5
7.66	0.48
7.88	0.44
8.04	0.41
9.25	0.46
9.45	0.52
9.63	0.60
9.84	0.66
10.03	0.72
13.00	0.86 (^{-}OB)
0.33	($HOB + {}^{-}OBH^{+}$)[a]

[a]Estimated; see text for details.

TABLE 3

Ground and excited state pK values for harmol acid–base equilibria

	pK_{a_1}	pK_{a_2}	pK_1	pK_2	pK_3	pK_4	pK_T
S_0	7.8	9.6	8.0[a]	8.2	9.4	9.1	0.23
S_1			13.6	6.2	4.4	11.9	−7.5

[a]This value is identified with the K_m value [13].

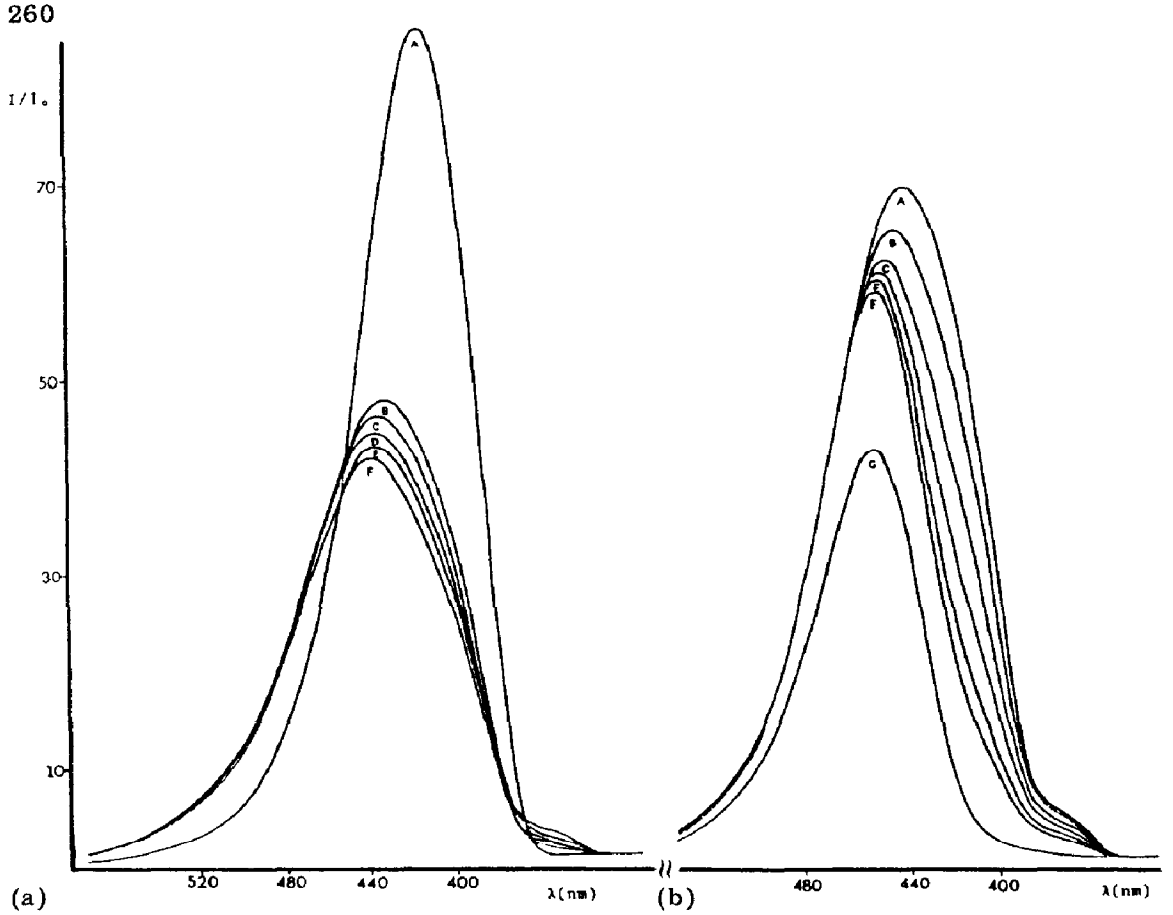

Fig. 4. Fluorescence spectra of harmol solutions ($C = 5 \times 10^{-5}$ M): (a) pH range 1.05 - 8.23 (curve A, pH 1.05; curve B, pH 7.47; curve C, pH 7.66; curve D, pH 7.88; curve E, pH 8.04; curve F, pH 8.26; λ_{ex} = 303 nm); (b) pH 9.05 - 13.00 (curve A, pH 9.05; curve B, pH 9.25; curve C, pH 9.45; curve D, pH 9.63; curve E, pH 9.84; curve F, pH 10.03; curve G, pH 13.00; λ_{ex} = 303 nm).

nm (the second isosbestic point in the absorption spectra) was chosen in order to guarantee that all of the molecular species arising from the acid–base equilibria of harmol would absorb.

At low pH values the spectrum shows an intense band that peaks at λ_{em} = 418 nm. This band decreases in intensity and is red shifted as the pH of the solutions increases. Consequently we conclude that this emission is due to a cationic species $HOBH^+$. At the opposite end of the pH range, *i.e.* at pH 13, an emission maximum is recorded at λ_{em} = 450 nm that arises from the anionic form ^-OB.

At intermediate pH values the fluorescence spectrum shows two important features: one moderately strong emission that peaks at λ_{em} = 440 nm and a very low intensity emission at λ_{em} = 360 nm. Also a shoulder near λ_{em} = 420 nm can be seen for pH values less than 9.5.

We conclude that three molecular species emit in the pH range 6 - 9.5. The shoulder at about 420 nm indicates the existence of appreciable concentrations of the acid form $HOBH^+$ in these media and consequently that the

equilibria described by K_1 and K_4 are displaced to the acid form in the first excited singlet.

The low intensity emission at λ_{em} = 360 nm can only be produced by the neutral species HOB. The absorption spectrum of the zwitterionic species shows its low energy maximum at longer wavelengths, and this weak emission disappears if the pH is changed. Consequently the peak at λ_{em} = 440 nm must correspond to the zwitterionic form $^{-}OBH^{+}$. In order to confirm these assignments we have carried out additional experiments in the adsorbed phase. As it is known, ionic molecular species absorb strongly on cellulose surfaces. We prepared a 10^{-3} M solution of harmol at pH 8.3 and submerged a clean strip of filter paper in it for a few minutes. The strip was stored over a desiccant in order to dry it; increasing the ionic strength by adding NaCl enhanced the adsorption of the ionic species but not of the neutral molecule. The dried strip was placed in the cell compartment and its fluorescence spectrum recorded. By exciting at λ = 380 nm (where the $^{-}OBH^{+}$ species fundamentally absorbs and there is some absorption from the $HOBH^{+}$ species) the spectrum shown in Fig. 5 was recorded. It can be seen that two peaks appear: one at about λ_{em} = 420 nm that arises from the cationic species $HOBH^{+}$ and one recorded at $\lambda_{em} \approx$ 440 nm that corresponds to the zwitterion $^{-}OBH^{+}$. On exciting at λ = 303 nm, there is no emission at λ_{em} = 360 nm in the adsorbed phase.

No additional emissions have been recorded at higher pH values. These emissions would correspond to species arising from the breaking of the N—H bond and consequently we can conclude that an acid–base equilibrium involving this bond does not occur in aqueous solutions in the pH range studied.

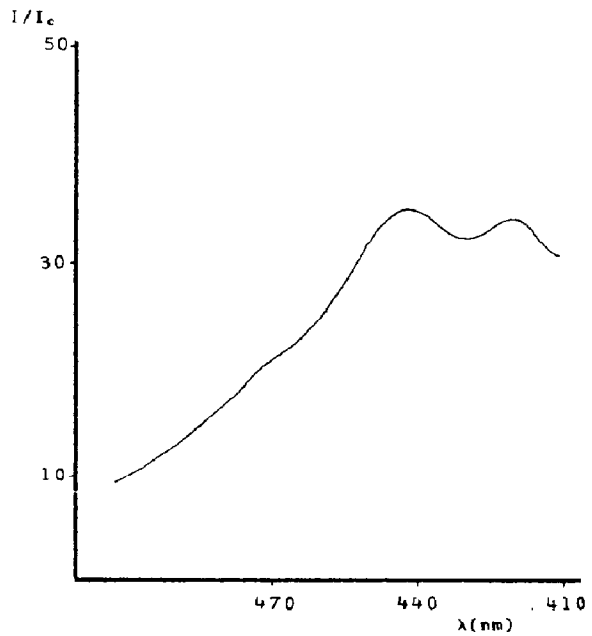

Fig. 5. Fluorescence spectrum of harmol in the adsorbed phase (pH 8.3; λ_{ex} = 380 nm).

All the emission maxima are summarized in Table 1. By applying the Förster cycle, all of the pK_a values of the first excited singlet state have been calculated. The results are summarized in Table 3.

4. Conclusions

From the pK_a values found for the ground state two features must be noted.

The four equilibrium constants K_1, K_2, K_3 and K_4 have very similar values. It seems very difficult to isolate $HOBH^+$ and HOB in solution, as was indicated by the absorption spectra. The tautomerism constant $K_T = 0.58$ indicates that the zwitterionic species has a lower concentration than the neutral species, but that the two acid–base centres, *i.e.* the phenolic OH and the pyridinic N^+—H, have similar acid–base properties, the N^+—H bond being slightly more acid than the H—O bond.

The situation changes markedly in the first singlet excited state. The O—H group seems much more acid than the N^+—H group. The concentration of HOB is negligible in any case, as has been observed from its emission, and consequently the tautomeric equilibrium is displaced towards the zwitterionic form. In view of the pK_3^* and K_T^* values, the observation of the weak emission due to the neutral form HOB indicates that the tautomeric equilibrium is not fully attained during the lifetime of the excited species. This behaviour agrees well with that observed for other nitrogen heterocycles [16 - 20] and phenolic systems.

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