SPECTROFLUOROMETRIC CHARACTERISTICS AND ACID–BASE EQUILIBRIA OF SERPENTINE

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Summary

The corrected excitation and fluorescence emission spectra of serpentine hydrogen tartrate in aqueous solutions of varying acidity and in several solvents are reported, and the quantum yields and fluorescence sensitivity have been calculated. The ground state pK_a have been determined from the acidity dependence of the absorption spectra and the excited state pK_a have been determined using the Förster–Weller equation.

1. Introduction

Serpentine (Oxayohimbanium,3,4,5,6,16,17-hexadehydro-16[methoxycarbonyl]-19-methyl-[19α]) is a Rauwolfia alkaloid with antihypertensive and sympatholytic properties. Moreover, this alkaloid selectively inhibits cancer cell DNA synthesis *in vitro* [1, 2].

The fluorescence of serpentine in various solvents [3 - 5] and the limit of detection in 0.1 N sulphuric acid have been reported previously. However, only uncorrected excitation and fluorescence spectra were given. Corrected spectra are very valuable for studying the fluorescence of solutions. They permit quantum yields, which are of central theoretical and practical importance, to be obtained.

In the present paper we report a spectrofluorometric study of serpentine hydrogen tartrate. Corrected excitation and fluorescence emission spectra in water, methanol, ethanol, acetonitrile and 5 M acetic acid have been obtained. Also, the wavelength of maximum excitation and maximum emission in these solvents have been found. The quantum yields in ethanol, 0.05 M sulphuric acid and 5 M acetic acid were determined. The fluorescence sensitivity in ethanol and the limits of detection in water and ethanol are given.

The ionization constants of pharmacologically active substances are extremely important to an understanding of the action of drugs. The proton dissociation constant of the indole ring has been evaluated by investigating

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the pH dependence of the absorption spectrum. The protonation of the indole nucleus in concentrated sulphuric acid solution is also discussed. Finally, we report the excited state pK_a calculated using the Förster–Weller equation [6].

2. Experimental details

2.1. Materials

Serpentine hydrogen tartrate was supplied by C.H. Boehringer Sohn Ingelheim. The solvents (Merck) were ethanol and methanol for fluorometry, acetonitrile for spectroscopy and reagent grade glacial acetic acid. The other chemicals employed were analytical grade.

2.2. Apparatus

Absorption spectra were recorded using a Perkin–Elmer Lambda 5 spectrophotometer. Fluorescence spectra were recorded using a Perkin–Elmer 650-40 spectrophotofluorometer. A Perkin–Elmer Data Processor 650-0178 was used to obtain corrected spectra. The wavelengths of excitation and emission were calibrated against the xenon line emission spectrum. The sensitivity and stability were checked by using the Raman band of distilled water. The pH values were measured directly with an accuracy to 0.01 pH units using a Radiometer 82 pH meter.

3. Results and discussion

The absorption spectra were recorded at an alkaloid concentration of 3×10^{-5} M. The spectra for aqueous solutions of various acidity are shown in Fig. 1.

The corrected excitation and emission spectra were recorded as relative quantum per unit wavelength interval *versus* wavelength. Rhodamine B was the standard employed for the quantum counter. The solutions contained 2×10^{-5} M serpentine hydrogen tartrate. Corrected emission spectra for aqueous solutions of various acidity are presented, as examples, in Fig. 2. The absorption, excitation and fluorescence maxima for the various solvents are compiled in Table 1.

The relationship between the relative fluorescence intensity at the emission maximum and concentration was investigated in ethanol and in water. The fluorescence is proportional to the concentration in the range 0.01 - 2 $\mu g\ ml^{-1}$. The limits of detection [7], for a confidence level of about 90%, were derived from the standard deviations of the blank measurements and from the sensitivities evaluated from the calibration curves (see Table 1).

The fluorescence quantum yields at 25 °C were determined by a comparison of the corrected emission spectra with the spectrum of a fluorescence standard (quinine bisulphate) using optically dilute solutions [8, 9],

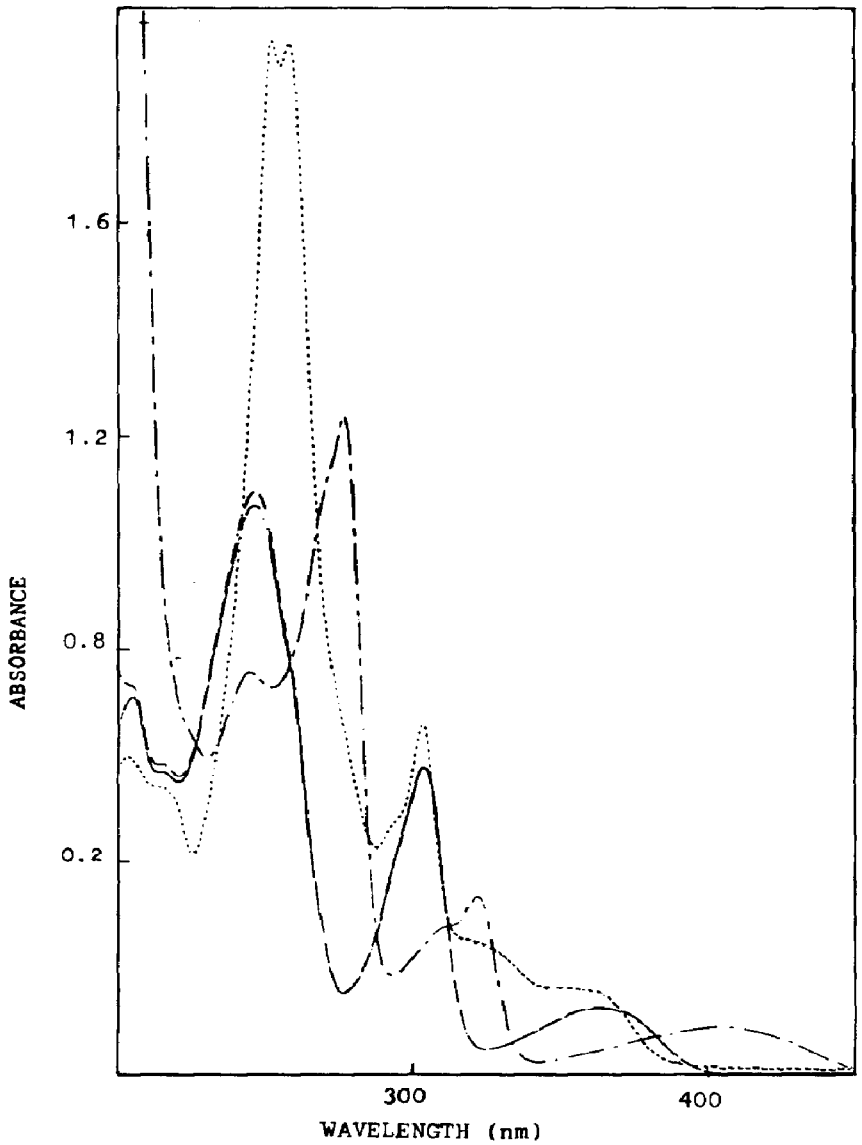

Fig. 1. Absorption spectrum of serpentine hydrogen tartrate (3×10^{-5} M) in H_2O (——), 0.05 M H_2SO_4 (– – –), 18 M H_2SO_4 (· · ·) and 0.02 M NaOH (– · –).

using the equation

$$Q_x = Q_r \frac{A_r(\lambda_r)}{A_x(\lambda_x)} \frac{I(\lambda_r)}{I(\lambda_x)} \frac{D_x}{D_r} \frac{{n_x}^2}{{n_r}^2} \quad (1)$$

where the subscripts r and x refer to the reference and the unknown solutions, Q is the quantum yield, $A(\lambda)$ is the absorbance per centimetre of the solution at the excited wavelength, $I(\lambda)$ is the relative intensity of the exciting light at the wavelength λ, D is the integrated area under the corrected emission spectrum, and n is the average refractive index of the solution at the maximum luminescence.

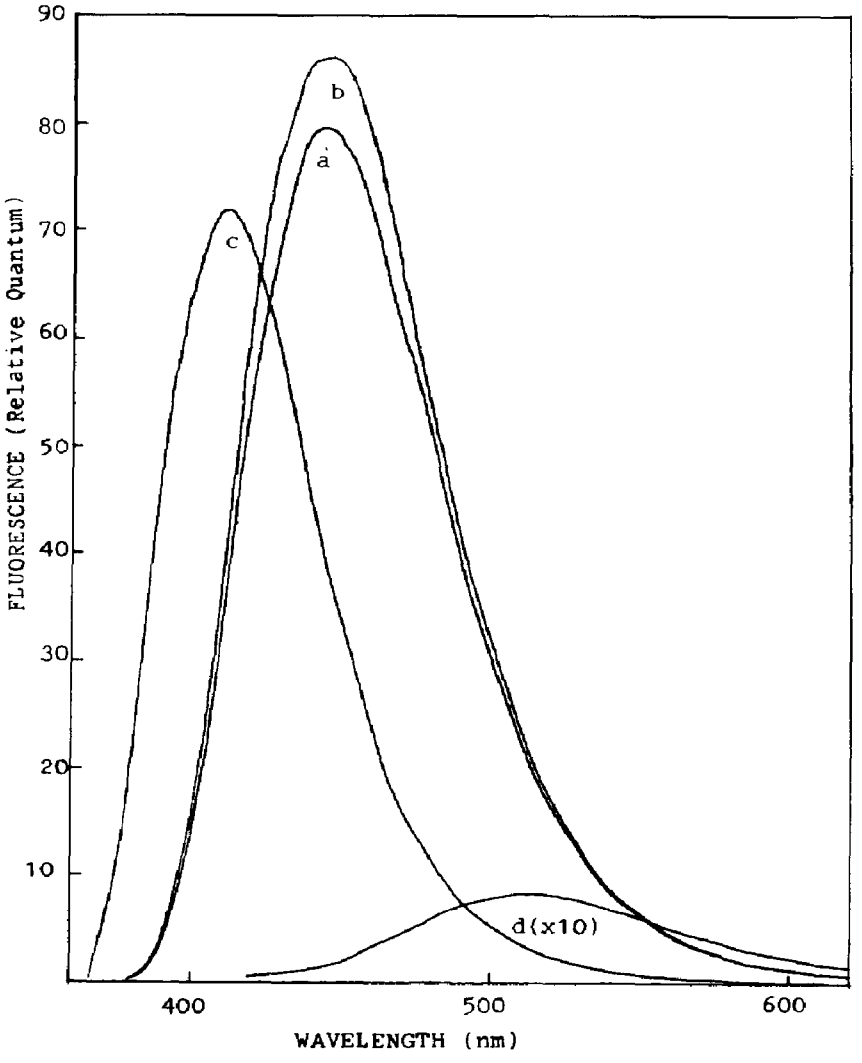

Fig. 2. Corrected fluorescence spectra of serpentine hydrogen tartrate (2×10^{-5} M): curve a, H_2O (λ_{exc} = 365 nm); curve b, 0.05 M H_2SO_4 (λ_{exc} = 365 nm); curve c, 18 M H_2SO_4 (λ_{exc} = 363 nm); curve d, 0.02 M NaOH (λ_{exc} = 405 nm).

The areas under the corrected emission spectra were calculated by connecting a microprocessor-based data acquisition system to the spectrofluorometer using an especially written program. In order to diminish errors due to reabsorption and re-emission, dilute solutions of the standard and the alkaloid with comparable absorbances of about 0.01 cm^{-1} were used. Also, the solutions were bubbled with nitrogen to avoid the quenching of dissolved oxygen.

The fluorescence emission spectra of the standard and the unknown solutions were measured with the same slit arrangement and at the same excitation wavelength (350 nm). Quinine bisulphate in 0.1 N sulphuric acid appears to be an appropriate standard [9]. Melhuish's value [10] of 0.546 at

TABLE 1

Absorption, excitation and fluorescence maxima, limits of detection and fluorescence quantum yields of serpentine hydrogen tartrate in aqueous solutions of various acidity and in several other solvents at 25 °C

λ_{max}^{abs} (nm)	λ_{max}^{exc} (nm)	λ_{max}^{flu} (nm)	*Limit of detection* (ng ml^{-1})	*Fluorescence quantum yields Q*
Water				
365	365	445	0.47	
306	306		0.05	
248	248			
Sulphuric acid (0.05 M)				
365	365	445		0.65
306	306			
248	248			
Sulphuric acid (18 M)				
363(sh)	363	415		
303	303			
259	255			
253				
Sodium hydroxide (0.02 M)				
405	405	520		
322	322			
276	276			
244				
Methanol				
368	368	445		
308	308			
252	250			
Ethanol				
368	368	445	0.47	0.63
308	308		0.05	
252	252			
Acetonitrile				
365	365	442		
306	306			
250	250			
Acetic acid (5 M)				
368	368	444		0.66
308	308			
260	260			

sh, shoulder.

25 °C was used for the absolute quantum yield of quinine bisulphate. The values of the refractive index were taken from ref. 11. The procedure was checked by determining the quantum yield of anthracene in ethanol. A value

of 0.27 was obtained which agrees well with the literature values [8]. The quantum yields are given in Table 1.

The absolute fluorescence sensitivities in ethanol were calculated as described by Parker and Rees [8]: D_{max}, the absorbance per centimetre for a concentration of 1 μg mol^{-1} at the absorption maximum at 365 nm, 0.012; QD_{max}, the fluorescence sensitivity for the whole fluorescence band, 7.56 × 10^{-3}; H, the half-bandwidth of the fluorescence spectrum, 0.41 μm^{-1}; QD_{max}/H, the fluorescence sensitivity for the peak fluorescence, 0.0184 μm.

The absorption and fluorescence spectra (Fig. 1 and Fig. 2 respectively) clearly indicate that in aqueous solution serpentine can exist as three different species. Their respective equilibria, with the equilibrium constants K_{a_1} and K_{a_2}, are shown in the scheme illustrated in Fig. 3.

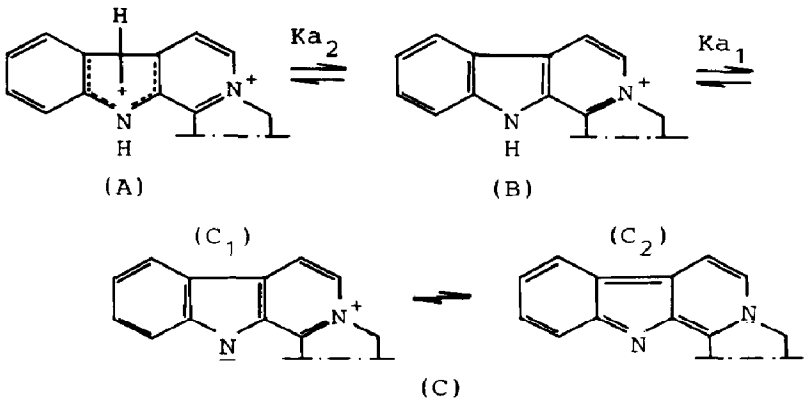

Fig. 3. Acid–base equilibria scheme for serpentine.

For the cation of the quaternary salt (B) the long wavelength absorption maximum occurs at 365 nm. This species shows a fluorescence maximum at 445 nm. This cation is present at pH values below 10, where it exists as the quaternary ammonium hydroxide. In more alkaline solutions, anhydro-base (C) absorption and emission are evident, with maxima at 405 nm and 520 nm respectively. C_1 and C_2 are canonical forms of a resonance hybrid [12]. These structures are not tautomeric forms. The presence of the C_2 form would explain the shift of the absorption and emission maxima to longer wavelengths. The fluorescence intensity of the alkaline solutions is very weak: this probably results from the quenching of the OH^- ion.

In strongly alkaline solutions a precipitate appears. Therefore, it is impossible to force the equilibrium B $\rightleftharpoons$ C so far to the right that all the serpentine is virtually in the form of the anhydro-base. For this reason, the molar absorption coefficient of C cannot be directly calculated from the Beer's law plot. However, it is possible to determine the K_{a_1} value by using the convergent straight lines method [13], because there is an acidity range in which only the B form is present. By plotting $1/(D - D_B)$ *versus* $\gamma[H^+]$ for various wavelengths (Fig. 4), the pK_{a_1} value can be obtained from the intercept of the convergent straight lines with the abscissa. D_B and D are the

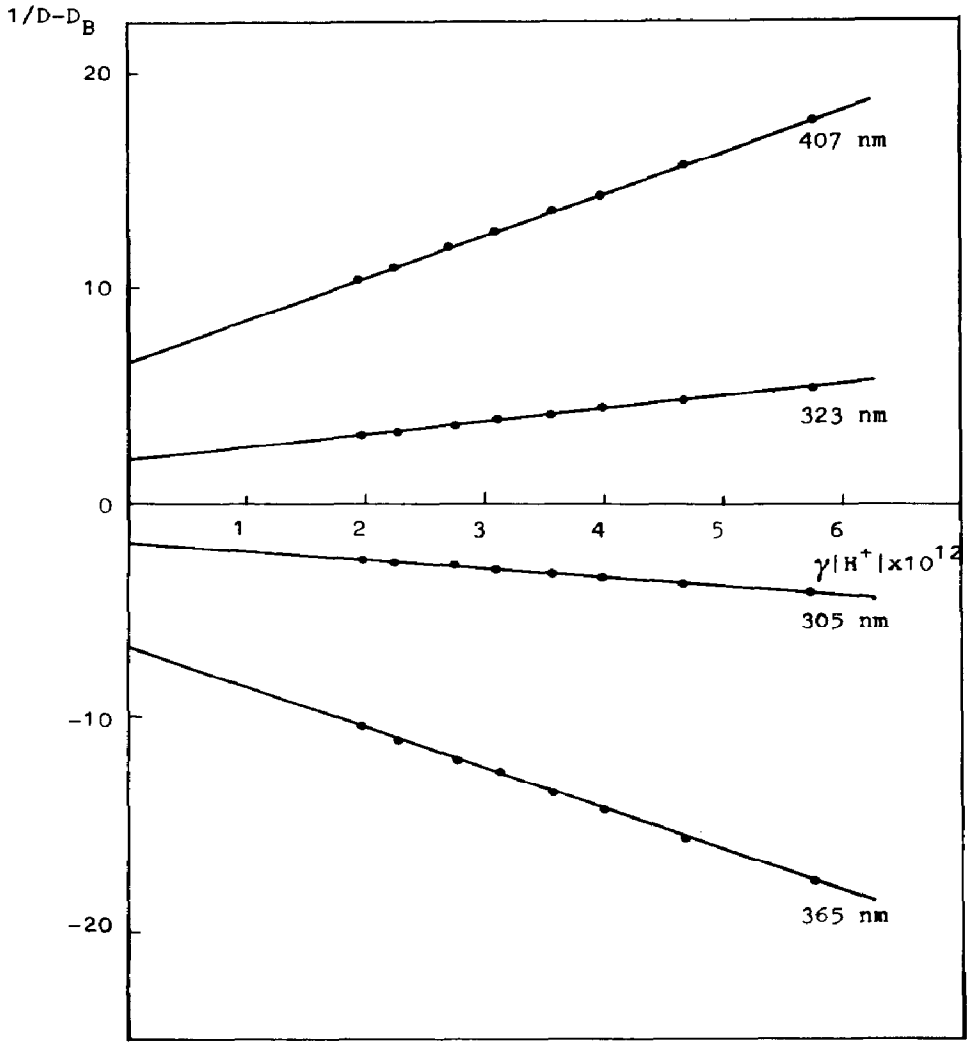

Fig. 4. Graphical method of convergent straight lines for evaluation of K_{a_1}.

absorbances of the B form and of mixtures of B and C respectively. The ionic strength was 0.02. The calculated thermodynamic pK_{a_1} value was 11.51 ± 0.05 at 25 °C. This value agrees well with that obtained by Wolfbeis *et al.* [14] for an *N*-methyl derivative of the harman cation. Schwarz and Schlitter [15] give the value 10.49 in 40% methanol, obtained by potentiometric titration.

In concentrated sulphuric acid solutions there is again a modification in the UV spectrum (Fig. 1). Two new absorption maxima appear, at 253 nm and 259 nm, which arise from the formation of green fluorescent serpentine cations. The fluorescence spectrum of this cation is shown in Fig. 2. The excitation and emission wavelengths are reported in Table 1.

These modifications may be explained by considering the indole ring to be protonated. The weakly basic character of the indole ring has long

been known [16, 17], but only in concentrated acid solutions. Since serpentine contains the indole nucleus in its structure, protonation can be expected in strong sulphuric acid solutions.

The pK_a of this protonation equilibrium was determined spectrophotometrically by measuring the absorbance changes of serpentine in concentrated sulphuric acid solutions at 257 nm. This wavelength was found to be the most suitable for the determination of the ionization ratio owing to the high absorbance changes on protonation.

Protonated serpentine is unstable in very concentrated sulphuric acid solutions as shown by the time dependence of the absorbance. Therefore, absorbances were determined by linear extrapolation. However, at acidities higher than about 16 M sulphuric acid it was very difficult to obtain extrapolated values of absorbances. For this reason we attempted to overcome these difficulties by use of the previously indicated convergent straight lines method [13], because it only requires the part of the titration curve before the half-protonation point which is readily accessible.

Since protonation occurs only in very strongly acidic media, the Hammett acidity function parameter or one of its variations must be invoked to provide the pK_a value. In this sense, it is interesting to note that indoles have a protonation pattern that is not consistent with the use of the Hammett acidity function. Proton magnetic resonance and UV spectra [16, 17] showed that protonation occurs mainly on C-3 in the indole ring and that the positive charge is centred on the nitrogen atom.

An acidity function H_I for indole protonation was introduced by Hinman and Lang [17]. However, it was not investigated over a sufficiently large range to accommodate our data. Since H_I values are proportional to the sulphuric acid molar concentration over much of the acidity range, the required H_I values were obtained by linear extrapolation.

According to the convergent straight lines method, $1/(D - D_B)$ were plotted against $1/h_I$, where D_B and D are the absorbances of the B form and of mixtures of A and B respectively and h_I = antilog$(-H_I)$. This plot gave a good straight line (Fig. 5) whose intercept on the abscissa gave a pK_{a_2} value of -11 ± 1. A similar plot for H_0 was not linear, which seems to confirm that the H_I function is the better measure of serpentine basicity.

Shifts in the long wavelength absorption band and in the fluorescence band of acidic or basic molecules, upon protonation or dissociation, can be related to the difference between the lowest excited singlet state pK_a^* and the ground state pK_a by the Förster–Weller equation [6]

$$pK_a^* - pK_a = \frac{0.625}{T}(\bar{\nu}_{Ba} - \bar{\nu}_{Ac}) \qquad (2)$$

where $\bar{\nu}_{Ac}$ and $\bar{\nu}_{Ba}$ are the average of the absorption and fluorescence maxima (in reciprocal centimetres) of the conjugate acid and the base respectively (taken as 0–0 transitions).

At 25 °C the results are $pK_{a_1}^* - pK_{a_1} = -6.2$ for species B and $pK_{a_2}^* - pK_{a_2} = -1.9$ for species A (scheme, Fig. 3). Both species are stronger acids

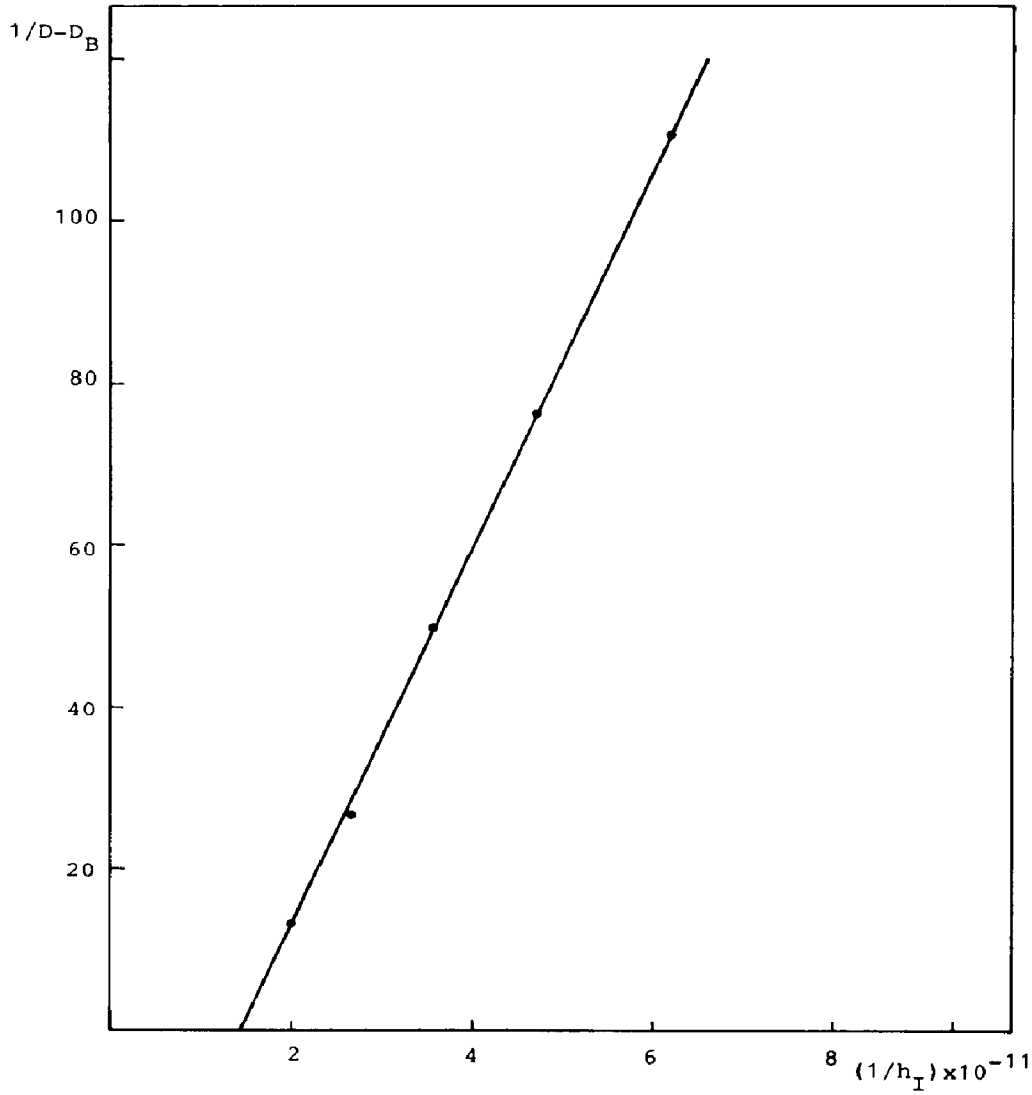

Fig. 5. Graphical method of convergent straight lines for evaluation of K_{a_2} at 257 nm.

in their S_1 states. In Table 2 the ground and excited pK_a for these species are compiled.

Although the prototropic equilibrium could be established in the lowest excited singlet state, the corresponding pK_a* cannot be determined by fluorescence titration. For B species the pK_a is in a pH region where the low concentrations of H_3O^+ and OH^- result in low overall rates of second-order protonation and proton abstraction [18]. Consequently, the fluorometric titration gives the ground state pK_a. For the A species, the fluorescence intensity in the sulphuric acid solutions was not stable, and furthermore the maximum acidity of the solutions used was not sufficiently high for the titration curve to be completed.

TABLE 2

Experimental ground and calculated lowest excited singlet state pK_a of serpentine hydrogen tartrate at 25 °C

Species	$pK_a(S_0)$	$\bar{\nu}_{abs}$ (cm^{-1})	$\bar{\nu}_{fl}$ (cm^{-1})	*0–0 transition*	$pK_a^*(S_1)$
A	−11 ± 1	27548	24096	25822	−12.9
B	11.51 ± 0.05	27393	22472	24935	5.3
C		24691	19231	21961	

4. Conclusions

The absorption, corrected excitation and fluorescence maxima of serpentine hydrogen tartrate in aqueous solutions of varying acidity and in different solvents are shown in Table 1.

The quantum yields at 25 °C in ethanol, 0.05 M sulphuric acid and 5 M acetic acid were 0.63, 0.65 and 0.66 respectively. The absolute fluorescence sensitivities in ethanol were calculated and are given in Section 3.

The absorption and fluorescence spectra indicate clearly that in aqueous solutions serpentine can exist as three different species (see scheme, Fig. 3): the cation of the quaternary salt (B), the anhydro-base (C) and the form with a protonated indole nucleus (A).

The ground and excited state pK_a values for the acid–base equilibria present in aqueous solutions of serpentine hydrogen tartrate are summarized in Table 2. It can be seen that the A and B species (scheme, Fig. 3) are stronger acids in their S_1 states than in their ground states.

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