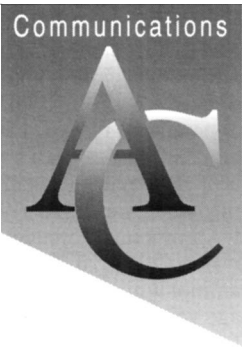

Use of Haematoxylin in the Spectrophotometric Determination of Alkaloids in Pharmaceuticals, Galenicals and Powdered Plants

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A simple and sensitive method for the determination of about fifteen alkaloids is described. The procedure is based on the reaction of the alkaloids with haematoxylin reagent in aqueous medium to give a red–violet colour (λ_{max} = 555 nm). Several variables affecting the colour development were studied and optimized. The method was used to determine 2–40 μg ml^{-1} of the final measured alkaloidal base solution. There was a considerable increase in the absorptivity, which was dependent on both the basicity (pK_a) and molar concentration of the alkaloid present. The simplicity of the method permits rapid analysis, suitable for routine quality control. The interference of about 28 substances which are commonly prescribed with alkaloids was studied. No interference due to additives commonly present in the pharmaceutical preparations was observed. The method could be applied for the analysis of alkaloids in pure and pharmaceutical dosage forms, galenicals and powdered plants.

Alkaloids occupy a prominent historical position among extensively employed medicaments. Accordingly, various approaches to their colorimetric analysis were reported. They include extraction by ion pairing with a dye anion,[1–4] quantitative precipitation as reineckate and the subsequent dissolution in acetone[5,6] and charge transfer complex formation with iodine and π-acceptors.[7–10] The British Pharmacopoeia (1993) specifies the extraction of individual alkaloids followed by acid–base titration and TLC check on foreign bases.[11] Some individual alkaloids were determined by measuring the developed colour after oxidation,[12] condensation[13] or nitration.[14]

Most plant alkaloids are derivatives of tertiary amines, while others contain primary, secondary or quaternary nitrogen. Their basicity therefore varies greatly, depending on which of the four types is represented. The pK_a values are 0–4 for very weak bases (purines), 4–7 for weak bases (papaverine, pilocarpine and reserpine) and 7–11 for medium strength bases (tropine, atropine, nicotine and emetine).[15] As most alkaloids are weakly UV-absorbing and the reported methods for their analysis are time-consuming, the development of a simple, rapid and sensitive procedure is required.

Haematoxylin has been used mainly for the analysis of metals,[16] in manufacturing of ink and chiefly as a stain in microscopy.[17] Oxidized haematoxylin has been used for the determination of some penicillins and cephalosporins.[18]

The aim of the present work is to develop a method suitable for the quantitative analysis of some of these alkaloidal bases, depending upon their pK_a values, using haematoxylin reagent. The method could be utilized for the determination of many alkaloids in pure and pharmaceutical formulations as well as in some plants and liquid extracts containing alkaloids.

Experimental

Apparatus

Uvidec-320 (Tokyo, Japan) and Perkin-Elmer 3B UV/VIS (Norwalk, CT, USA) spectrophotometers with 1 cm quartz cells were used. All volumetric measurements were made with standard glassware.

Reagents and Materials

All solvents and reagents were of analytical-reagent grade. Doubly distilled water was used throughout.

Haematoxylin. Obtained from Aldrich, Poole, Dorset, UK; 0.2% (m/v) is prepared fresh daily by dissolving 100 mg in 2 ml of 0.5% (m/v) boric acid and diluting to 50 ml with doubly distilled water.

Boric acid. Solutions, 0.5 and 0.05% (m/v) were prepared in distilled water.

Alkaloids. Tropine, quinine, ephedrine, nicotine and ajmaline bases; brucine trihydrate; emetine hydrochloride; sparteine, (L)-lobeline and strychnine sulfates; (DL)-homatropine and hyoscine hydrobromides; codeine phosphate and physostigmine salicylate were obtained from different manufacturers and were used as working standards.

Formulations

The following available commercial preparations were analysed: (1) Ephedrine HCl tablets (Kahira Pharmaceuticals and Chemicals, Cairo, Egypt), containing 0.5 grain of ephedrine HCl per tablet; (2) Efanol tablets (Memphis, Cairo, Egypt), containing ephedrine HCl (20 mg), chlorpheniramine maleate (2 mg), dihydroxypropyl theophylline (250 mg) and phenobarbitone (10 mg) per tablet; (3) Ephedrine sulfate ampoules (Misr, Cairo, Egypt), containing ephedrine sulfate (50 mg) per ampoule; (4) Asthmolase tablets (Memphis), containing ephedrine HCl (20 mg), papaverine HCl (10 mg), homatropine (0.5 mg), caffeine (20 mg) and phenobarbitone (20 mg); (5) Tepedrine tablets (Misr), containing ephedrine HCl (25 mg), theophylline (120 mg) and phenobarbitone (8 mg); (6) Atropine sulfate ampoules (Nile, Cairo, Egypt), containing atropine sulfate (1 mg ml^{-1}); (7) Isoptoatropine eye drops (Alcon, Puurs, Belgium), contain atropine sulfate (1%), benzalkonium chloride (0.01%) and hydroxypropyl methyl cellulose (0.5%); (8) Bellacid tablets (CID, Cairo, Egypt), containing extract of *Belladona siccum* (0.01 g) and phenobarbitone (0.02 g); (9) Supergine ampoules (Memphis), containing homatropine

methylbromide (2.5 mg), dipyrone (1 g) and papaverine (50 mg); (10) Codacetine tablets (Kahira Pharmaceuticals and Chemicals), containing salicylamide (300 mg), paracetamol (250 mg) and codeine phosphate (10 mg); (11) Vegaskine tablets (Alex, Alexandria, Egypt), containing acetyl salicylic acid (300 mg), paracetamol (200 mg) and codeine phosphate (10 mg); (12) Buscopan ampoules (CID), containing hyoscine butyl bromide (20 mg); and (13) Butacid tablets (CID), containing hyoscine butyl bromide (10 mg).

Liquid extracts

Nux vomica (CID), Belladonna (Paul Mugenburg, Germany), Hyoscyamus (Memphis), Ipecacuanha (Arab Drug, Egypt) were used.

Powdered plants

Cinchona bark consists of the dried bark of *Cinchona succirubra* Pavon, family Rubiaceae; Rauwolfia consists of the dried rhizome and roots of *Rauwolfia serpentina*, family Apocynaceae; and cigarette samples were obtained from Eastern Company, Egypt.

Procedure

Standard solutions

Dissolve a calculated amount of alkaloidal base in absolute ethanol and dilute quantitatively to 0.02–0.4 mg ml^{-1}. For alkaloidal salts, dissolve calculated amounts in about 20 ml of distilled water and transfer to a 100 ml separating funnel. Add 2.0 ml of 33% ammonia solution and extract with three 25 ml portions of chloroform, passing the separated organic layers through 5 g of anhydrous sodium sulfate supported in a small funnel. Evaporate chloroform, dissolve the residue in 10 ml of ethanol in a 100 ml calibrated flask and make up to the mark with the same solvent.

Sample solutions

Liquid preparations (ampoules and eye drops). Transfer aliquot portions equivalent to 50 mg of alkaloidal base into 50 ml of distilled water in a 100 ml separating funnel and continue as described above for standard solutions.

Tablets of alkaloidal salts. Place an accurately weighed amount equivalent to 50 mg alkaloidal base from composite of 20 powdered tablets in 50 ml of distilled water in a 100 ml separating funnel and continue as described above for standard solutions.

Galenical preparations

Evaporate 10 ml of the liquid extract to dryness. Extract the residue with water acidified with sulfuric acid (1 + 100). Transfer the aqueous extract into a 100 ml separating funnel through a filter paper and wash with 2 ml of dilute sulfuric acid (1 + 100), add the aqueous washings to the mother liquor and render it alkaline to litmus with 33% ammonium hydroxide solution (≈ 2 ml). Shake with successive portions of chloroform until the alkaloids are completely extracted (3 × 25 ml). Evaporate the combined chloroformic extract on a water-bath to dryness and extract with ethanol in a 100 ml calibrated flask. Dilute quantitatively 1.0 ml of this solution to 100 ml with ethanol.

Powdered plants

A 20 g sample of cinchona bark or rauwolfia root powders, dried and ground to 40 mesh was heated under reflux with 100 ml of 70% ethanol for 2 h. The extract was concentrated to 25 ml. Extract with water acidified with sulfuric acid (1 + 100) and continue as described above under Galenical preparations beginning from 'Transfer the aqueous extract . . . '.

Cigarette Fillers

Remove sufficient filler from tobacco product. Weigh 2 g of the filler into a tared 250 ml Erlenmeyer flask, add 100 ml of 0.05 mol l^{-1} sulfuric acid and place on a shaker for about 15 min. Transfer into a separating funnel through a filter paper. Render alkaline to litmus with 33% ammonium hydroxide solution and continue as described above under Galenical preparations.

General procedure

Pipette 1.0 ml of the standard or sample solutions into a dry 10 ml calibrated flask, add 1.0 ml of haematoxylin reagent. Allow to stand for 30 min at 25 ± 5 °C, add 1 ml of 0.05% boric acid solution. Complete to the mark with distilled water and measure the absorbance at 555 nm against a reagent blank.

Results and Discussion

Addition of haematoxylin aqueous solution to an aqueous alcoholic solution of any of the investigated alkaloidal bases

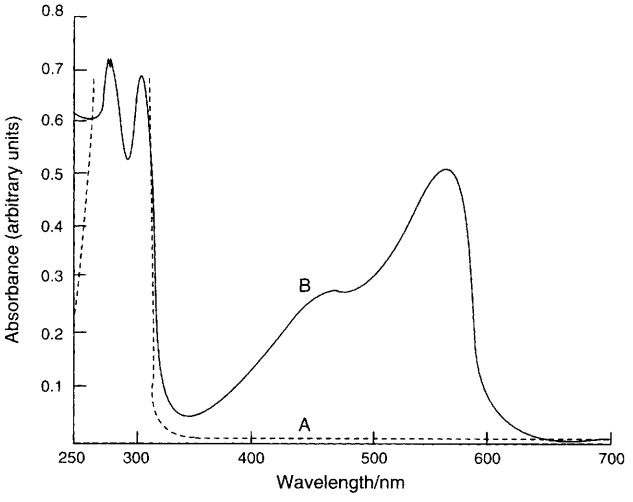


Fig. 1 Absorption spectra of A, 0.2 mg ml^{-1} haematoxylin and B, its reaction with atropine (16.5 μg ml^{-1}).

Table 1 Some spectral characteristics of the investigated alkaloids

	Alkaloidal base	Linear range/ μg ml^{-1}	Intercept (*a*)	Slope (*b*)	*r*
1	Tropine	2–12	−0.0173	0.0716	0.9996
2	Atropine	2–20	−0.0064	0.0346	0.9952
3	Homatropine	5–25	−0.0180	0.0370	0.9996
4	Ephedrine	2–15	0.0060	0.0540	0.9999
5	Sparteine	2–20	−0.0060	0.0400	0.9992
6	Quinine	5–25	−0.0090	0.0273	0.9957
7	Emetine	5–40	−0.0040	0.0184	0.9970
8	Ajmaline	5–35	−0.0051	0.0263	0.9961
9	Codeine	2–30	0.0020	0.0283	0.9996
10	Brucine	5–40	−0.0050	0.0200	0.9985
11	Strychnine	5–40	0.0047	0.0253	0.9985
12	Nicotine	2–15	−0.0103	0.0525	0.9986
13	Physostigmine	5–30	0.0150	0.0300	0.9997
14	Hyoscine	5–30	0.0020	0.0223	0.9990
15	Lobeline	5–25	−0.0064	0.0375	0.9994

yields an intense red–violet colour. The absorption spectrum of the resulting chromogen is characterized by the formation of a new band (λ_{max} = 555 nm) which was not found in the spectrum of either the reagent or the alkaloids (Fig. 1). A fresh solution of haematoxylin possesses an absorption band at 290 nm.

The stability of the reagent was investigated. Haematoxylin was found to be unstable in water as its solution darkens on standing. Trials were made for the stabilization of its aqueous solution and it was found that 2.0 ml of 0.5% (m/v) boric acid solution per 50 ml reagent solution is a suitable medium for this purpose (haematoxylin borate complex).

The effect of diluting solvents was studied. As assay solvent, water afforded maximum sensitivity and stability. Methanol, ethanol, propan-1-ol, propan-2-ol and acetone were not suitable as diluting solvents because they gave lower absorbance readings. Halogenated and other water immiscible solvents were found to be unsuitable as the formed chromogen is not extractable into these solvents.

The stability of the colour was investigated. Maximum absorbance readings at 555 nm were achieved after 30 min duration followed by quenching the reaction with 1.0 ml of 0.05% boric acid solution. Stability was then manifested for more than 30 min at 25 ± 5 °C.

The effect of reagent concentration was investigated. The colour develops strongly as the amount of haematoxylin reagent in the reaction mixture was increased from 0.5 to 2.0 ml of 0.2% solution. Therefore, 1.0 ml was used throughout the experimental work.

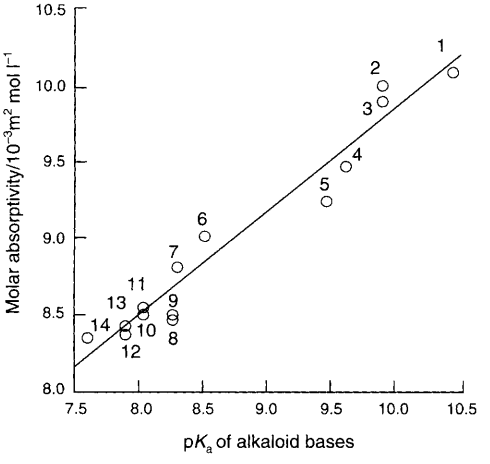


Fig. 2 Molar absorptivity *versus* pK_a values of haemotoxylin reaction products with alkaloids. (pK_a values from ref. 19 except for compound 1 from ref. 20 and 5 from ref. 17). Compound numbers refer to Table 1.

Under the specified reaction conditions, Beer's law was obeyed for 2–40 μg ml^{-1} of the final assayed solution. Slopes, intercepts and correlation coefficients are given in Table 1.

The mean of six replicate analyses of the different investigated alkaloids gave *s* values of 1.20–1.90%. This level of precision is adequate for the quality control analysis of pharmaceutical preparations and natural products.

The sensitivity of the assay, expressed as ε-values varies with different alkaloids in a regular pattern which was found dependent upon the pK_a values of these alkaloids, Fig. 2. Regression analysis of the above correlation by the method of least squares afforded r = 0.9801 and a regression equation of ε = 3063.8 + 679.9 pK_a From this equation, ε for the coloured

Table 2 Analysis of certain alkaloids in the presence of some coformulated ingredients

Ingredients*	Amount added/mg	Recovery of alkaloid (%) ± s†
Phenobarbitone[a,c,e,h]	400	98.44 ± 1.46
Dipyrone[a,c,h]	250	100.58 ± 1.57
Salicylamide[c,e]	300	99.27 ± 1.85
Cetrimide[e]	2	104.17 ± 0.98
Hydroxypropylmethylcellulose[a,h]	500	99.03 ± 1.39
Acetylsalicylic acid[c]	300	98.59 ± 1.41
Paracetamol[c]	250	100.35 ± 1.06
Dexamethasone[e]	1	103.55 ± 1.60
Sodium benzoate[e]	15	101.10 ± 1.11
Sodium citrate[a]	10	101.31 ± 1.61
Sodium salicylate[e]	5	98.78 ± 1.23
Sodium penicillin G[e]	10	99.27 ± 0.98
Sulfadimidine[h]	150	99.17 ± 1.53
Sulfagaunidine[h]	1500	139.89 ± 1.67
Sulfanilamide[h]	400	98.34 ± 1.25
Vitamin B_1[h]	3	96.95 ± 1.81
Vitamin B_2[h]	1	100.42 ± 0.83
Vitamin B_6[h]	1	97.65 ± 1.11
Nicotinamide[h]	10	99.03 ± 1.39
Vitamin C[e]	10	100.49 ± 1.48
Naphazoline nitrate[e]	125	149.50 ± 1.85
Nikethamide[e]	250	100.49 ± 0.86
Piperazine hydrate[a]	1800	300.29 ± 1.46
Menthol[e]	50	98.78 ± 1.35
Diiodohydroxyquinoline[h]	13	101.11 ± 1.39
Bismuth carbonate[h]	150	99.17 ± 1.67
Kaolin[h]	1500	100.83 ± 1.11
Potassium bromide[a]	400	102.49 ± 1.61

*[a,c,e,h] are alkaloids atropine (0.5 mg), codeine (15 mg), ephedrine (50 mg), homatropine (2000 mg), respectively, added to the above ingredients. † n = 3

Table 3 Analysis of some pharmaceutical dosage forms containing alkaloids

Dosage forms*	Label claim/mg	Found (%) ± s†	Added/ mg	Recovery (%) ± s†
Atropine sulfate ampoules	1	99.91 ± 1.72	1	100.80 ± 1.46
Isoptoatropine eye drops	1%	101.93 ± 1.78	1%	100.98 ± 1.90
Bellacid tablets	10‡	100.75 ± 2.01	1	99.20 ± 1.70
Ephedrine tablets	32.5	98.62 ± 1.58	32.5	98.67 ± 1.85
Efanol tablets	20	99.91 ± 2.27	20	99.30 ± 1.72
Ephedrine sulphate ampoules	50	98.90 ± 1.80	50	100.50 ± 1.72
Asthmolase tablets	20	100.74 ± 1.85	20	99.27 ± 1.48
Tepedrine tablets	25	99.27 ± 1.23	25	100.29 ± 0.98
Supergine ampoules	25	99.03 ± 1.67	25	100.83 ± 1.11
Codacetine tablets	10	98.60 ± 1.41	10	100.70 ± 1.58
Vegaskine tablets	10	99.12 ± 0.72	10	101.23 ± 1.41
Buscopan ampoules	20	100.44 ± 1.18	20	99.11 ± 1.78
Butacid tablets	10	98.96 ± 1.63	10	100.89 ± 1.04

* See text for detailed composition. † n = 4. ‡ As extract Belladona siccum and calculated as atropine.

product of an alkaloid with the reagent could be predicted from its corresponding pK_a value.

Specificity and Interference

The proposed procedure has the advantage that the assay is performed at 555 nm in the visible region away from UV-absorbing interferents that might be co-extracted from dosage forms, liquid extracts or powdered plants.

The failure of papaverine, reserpine, pilocarpine, colchicine and ergotamine to interact with the reagent is certainly related to their weak basicity (pK_a 6.4, 6.6, 6.9, 1.7 and 6.3, respectively). Purine bases: caffeine, theobromine and theophylline gave a negative response under the specified reaction conditions: these compounds have very low pK_a values and actually behave as weak acids.[21] Table 2 shows the results obtained from the analysis of some of the investigated alkaloids in the presence of ingredients commonly co-formulated with them. Interference from acidic substances could be eliminated by extraction at alkaline pH but highly basic lipophilic compounds, if present, would interfere with the analysis.

Analysis of Pharmaceutical Preparations

The strongly red-shifted band combined with the high intensity of absorption and the very low reagent background makes this method suitable for routine alkaloid analysis with minimum interference. The proposed procedure was applied to assay a number of commercial preparations containing alkaloids. Common ingredients of formulations such as tablet excipients were found not to interfere in the method when following the proper extraction procedure. The results in Table 3 indicate high accuracy and confirm the suitability of the proposed procedure for the analysis of the investigated alkaloids in the micro range. In tablets containing phenobarbitone (Efanol, Asthmolase and

Table 4 Assay of alkaloids in some galenicals

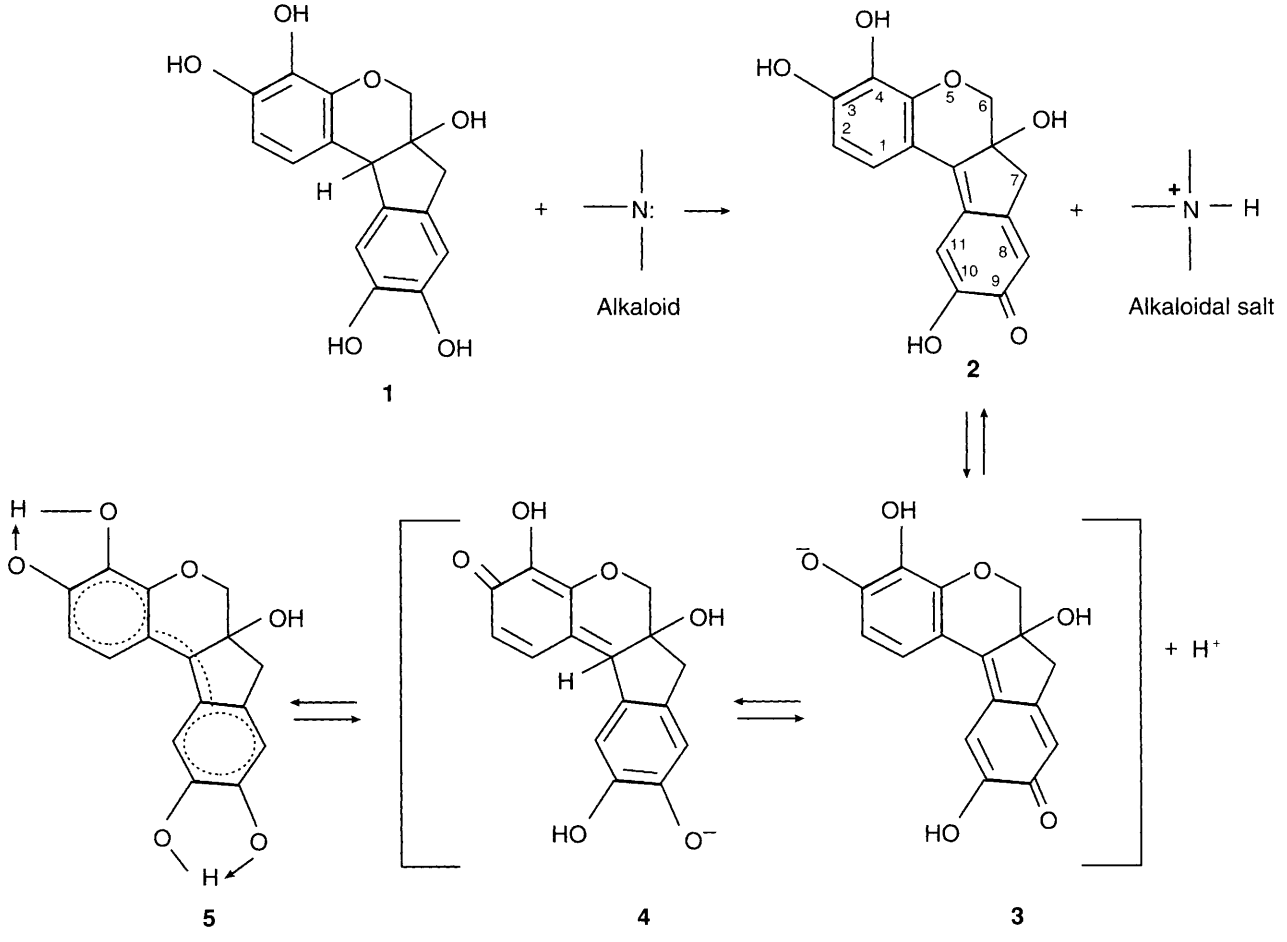
	Amount/g			Recovery
Liquid extract	Found	Reported*	Added	(%) ± s†
Nux Vomica	2.40‡	2.45–2.55	1.0	98.80 ± 1.50
Ipecacuanha	2.03§	1.95–2.05	2.0	99.20 ± 1.20
Belladonna	0.35¶	0.27–0.33	0.4	97.60 ± 1.70
Hyoscyamus	0.90¶	0.95–1.05	1.0	97.50 ± 1.90

* Ref. 21 and 22. † n = 3. ‡ Calculated as strychnine. § Calculated as emetine. ¶ Calculated as atropine.

Table 5 Assay of alkaloids in some powdered plants

	Amount/g			Recovery
Plant	Found	Reported	Added	(%), ± s*
Cinchona bark	6.36†	5–10‡	6.00	97.20 ± 2.30
Rauwolfia root	0.85§	0.7–2.4‡	0.85	97.80 ± 1.70
Cigarette fillers	p.22¶	0.17‖	0.20	98.10 ± 2.00

* n = 3. † Calculated as quinine. ‡ Ref. 22. § Calculated as ajmaline (non reserpine alkaloids). ¶ Calculated as nicotine. ‖ Ref. 23.



Scheme 1

Tepedrine tablets), the extraction at the alkaline pH was sufficient to separate the acidic phenobarbitone from the basic alkaloids. Chloropheniramine maleate, in spite of its considerable basicity, was found not to interfere with the analysis of Efanol tablets. This could be attributed to its low content relative to ephedrine content as well as the higher solubility of chloropheniramine base in aqueous phase. The data suggest good recoveries of added standards.

Analysis of Galenical Preparations and Powdered Plants

Table 4 gives the alkaloid contents of several liquid extracts analysed by the proposed method. The values were found to be consistent with the reported concentration ranges of these alkaloids.[21,22] In addition, analysis of alkaloids in some powdered plants could be also carried out. Table 5 shows the total alkaloid contents in cinchona bark, rauwolfia root and in cigarette fillers. No interference was observed from the presence of the very complex matrix of the plants. The accuracy of the method was confirmed by the good recovery of added alkaloids to the respective liquid extract.

Reaction Mechanism

Haematoxylin (**1**) has been previously oxidized to give haematein (**2**).[24,25] Since the formation of haematein is greatly facilitated by alkaline conditions, it seems likely that the removal of hydrogen is involved and it is suggested that alkaloids are the essential species responsible for the hydrogen acceptance. In this respect, the hydroxy group at position 9 of haematoxylin was oxidized to the corresponding keto derivative. The second stage is the ionization of the produced haematein which corresponds to the appearance of the 555 nm peak. The ionized haematein will possess two possible resonance forms (**3**, **4**) and these account for the intense colour of the ionic solution. The actual structure of the haematein ion will approximate to something between the two structures (**3**) and (**4**) and will be similar to (**5**). Scheme 1 shows the possible reaction pathway.

Conclusion

The proposed method is simpler, less time-consuming and more sensitive than the official titrimetric method, it could be applied for the routine quality control analysis of the investigated alkaloids in pure, pharmaceutical and galenical forms as well as in some powdered plants after adopting a suitable extraction procedure. The method is particularly recommended for assaying the tropine alkaloids and other weak UV-absorbing alkaloids such as ephedrine and sparteine. The proposed procedure could be considered specific for alkaloids having pK_a values greater than 7.6. However, it must be considered non-specific with regard to differentiation between them. These shortcomings do not affect the utility of the method in routine analysis and content uniformity determination of singly prescribed alkaloids. The disadvantages may also be overcome by coupling the proposed method to a suitable separation procedure. The enhanced sensitivity of the method allows for additional handling without risk of increasing errors.

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