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Note

Use of absorbance ratios in densitometric measurements for the characterization and identification of natural products of pharmacological interest

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Developments in densitometric measurement techniques have greatly enhanced their value in the analysis of natural products such as alkaloids[1,2], coumarins[3,4], saponins[5] and iridoids[5]. The improvements in detector systems (*e.g.*, multiwavelength ultraviolet detectors) allow the unequivocal identification of various components.

This paper adresses the feasibility of the rapid and reliable identification of some common natural products by their characteristic R_F values, absorbance ratios and constants obtained by the use of a densitometer.

EXPERIMENTAL

Apparatus

A Shimadzu (Kyoto, Japan) CS-930 thin-layer chromatographic (TLC) scanning photodensitometer of high sensitivity and precision was used. Precoated silica gel G glass TLC plates were obtained from E. Merck (Darmstadt, F.R.G.).

Materials

Pharmaceutical-grade compounds (Table I) were checked according to different Pharmacopoeias[5–10]. Solutions in methanol or chloroform (1 mg/ml) were prepared and were stable at 20°C for at least 2 months.

The following developing systems were used: ethyl acetate–methanol–water (100:13.5:10), toluene–ethyl acetate (93:7), chloroform–diethylamine (90:10), ethanol–acetic acid–water (60:30:10), methanol–ammonia solution (100:1.5), ethyl acetate–formic acid–acetic acid–water (100:11:11:27) and toluene–ethyl acetate–diethylamine (70:20:10).

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Procedure

Compounds representing different chemical classes were selected. Sample solutions contained 1 mg/ml of solute in methanol or chloroform. The sample size varried from 0.5 to 5 μl, depending on the compound.

The sample solutions were spotted side-by-side on the origin. The plates were developed for a distance of 15 cm using the developing systems listed in Table III. The absorbances of all compounds were recorded at 220 and 254 nm and the absorbance ratios (K_1) were calculated. The compounds may be identified primarily by their K_1 values (Table I), then from measurements at the wavelength of maximum absorption for each compound the K_2 ratio were determined (Table II) to confirm the compound identities. Each compound was chromatographed as six separate spots on one plate in order to verify the reproducibility and precision.

RESULTS AND DISCUSSION

Traditionally, the identification and quantification of a solute separated by TLC and monitored with a UV detector at a single wavelength are based on the R_F values[11]. However, if a solute is monitored at several wavelengths, the absorbances can be very different and reflect the UV-absorbing characteristics of the compound. Table III illustrates the results obtained by such a procedure after chromatographic development.

By selecting one of the wavelengths as a reference, absorbance ratios can be calculated. For colchicine, for instance 220 nm is selected as a reference wavelength. The calculated absorbance ratios will be the areas under the peaks at 254 and 220 nm (K_1) and 243 and 220 nm (K_2).

TABLE I

DATA OBTAINED BY CALCULATION FROM DENSITOMETRIC MEASUREMENT AND ARRANGED IN INCREASING ORDER OF (K_1) VALUES

K_1 = absorbance ratio, 254/220 nm (areas under the peaks).

Compound	K_1	Compound	K_1
Pilocarpine	0.028	Theobromine	1.32
Cinchonine	0.11	Theophylline	1.35
Cinchonidine	0.13	Harmaline	1.58
Yohimbine	0.30	Caffeic acid	1.60
Quinine	0.35	Quercetin	1.78
Eugenol	0.35	Berberine	1.81
Emetine	0.36	Psoralin	1.85
Codeine	0.37	Colchicine	1.95
Quinidine	0.38	Chlorogenic acid	1.95
Morphine	0.49	Anisaldehyde	2.41
Thymol	0.66	Papaverine	2.61
Heroin	0.71	Strychnine	2.93
Caffeine	0.99	Eserine	3.30
Methyl salicylate	0.99	Harmine	3.67
Brucine	1.01	α-Santonin	4.94
Vanillin	1.16		

TABLE II

DATA OBTAINED BY CALCULATION FROM DENSITOMETRIC MEASUREMENTS AND ARRANGED IN INCREASING ORDER OF K_2 VALUES

K_2 = absorbance ratio, $\lambda_{max.}/220$ nm.

Compound	$\lambda_{max.}{}^{a}$ (nm)	K_2	Compound	$\lambda_{max.}$ (nm)	K_2
Morphine	288	0.35	Colchicine	243	2.25
Cinchonine	303	0.41	Methyl salicylate	312	2.40
Codeine	290	0.41	Berberine	275	2.87
Cinchonidine	303	0.48	Caffeine	272	3.01
Heroin	282	0.85	Strychnine	262	3.04
Yohimbine	282	0.86	Quercetin	270	3.10
Thymol	278	1.08	Papaverine	247	3.50
Eugenol	283	1.08	Theophylline	273	3.76
Emetine	287	1.16	Theobromine	277	3.94
Quinine	238	1.17	Harmine	325	4.45
Psoralin	335	1.55	Caffeic acid	327	4.60
Quinidine	238	1.58	Anisaldehyde	297	5.50
Eserine	315	1.60	Chlorogenic acid	325	6.31
Harmaline	263	1.61	Pilocarpine[b]	—	—
Brucine	270	1.70	α-Santonin[b]	—	—
Vanillin	330	2.15			

[a] $\lambda_{max.}$ was measured directly *in situ* with the TLC scanner.

[b] Compounds with no maxima other than 254 nm.

Under controlled conditions, the UV absorption spectrum is characteristic for a specific compound, and hence the absorbance ratios derived from it also characterize the compound. These absorbance ratios can be used in conjunction with R_F data to identify the compound (Table III).

It has also been found that the two absorbance ratios (*i.e.*, monitoring at three wavelengths) gave adequate information to discriminate and identify the compounds examined, even those displaying very similar absorbance profiles, *e.g.*, purine bases (caffeine, theophylline, theobromine), phenanthrene alkaloids (morphine, codeine) and quinoline alkaloids (quinine, quinidine, cinchonine, cinchonidine) (Tables 1–3).

For selective and final identification of a compound, a wavelength of maximum absorption should be used (Table II). Classification of the compounds tested into five groups according to the first absorbance ratio K_1 (254/220 nm) (Table I), *e.g.*, (A) 0–0.13, (B) 0.30–0.49, (C) 0.66–1.00, (D) 1.00–1.96 and (E) 2.00 and above, will give the primary identification of groups. The second measurement at $\lambda_{max.}$ and calculation of the second ratio K_2 ($\lambda_{max.}/220$ nm) (Table II) will give the complete identification of the compound tested.

Another classification of the compounds into groups can be done according to the K_2 values by arranging them in increasing order. However, we prefer to start with K_1 as it represents a general and primary measurement at two fixed wavelengths (220 and 254 nm) whereas K_2 involves different wavelengths according to the compound tested.

The composition of the solvent systems was varied to achieve optimum

TABLE III

DATA OBTAINED BY CALCULATION FROM DENSITOMETRIC MEASUREMENT AND CLASSIFIED ACCORDING TO DEVELOPING SOLVENT

Compound	K_1	K_2	A^a	R_F	B^b	M.W.	C^c
Group I: developing solvent ethyl acetate–methanol–water (100:13.5:10)[12]							
Caffeine	0.99	3.01	2.98	0.46	15.43	194	89.2
Theobromine	1.32	3.96	5.23	0.36	6.88	180	64.8
Theophylline	1.35	3.76	5.07	0.53	10.45	180	95.4
Colchinine	1.95	2.25	4.38	0.29	6.60	399	115.7
Group II: developing solvent toluene–ethyl acetate (93:7)[12]							
Eugenol	0.35	1.08	0.37	0.47	127	164	77.0
Thymol	0.66	1.08	0.71	0.52	73.2	150	78.0
Anisaldehyde	2.41	5.50	13.25	0.10	0.7	136	13.6
Methyl salicylate	0.99	2.40	2.37	0.82	34.6	152	124.6
Vanillin	1.16	2.15	2.49	0.25	10.0	152	38.0
Psoralen	1.85	1.55	2.86	0.30	10.5	186	55.8
α-Santonin	4.94	–	–	0.10	–	246	24.6
Group III: developing solvent chloroform–diethylamine (90:10)[12]							
Quinine	0.35	1.17	0.40	0.14	35.0	324	45.3
Quinidine	0.38	1.58	0.60	0.30	50.0	324	97.2
Cinchonine	0.11	0.41	0.04	0.38	950.0	294	111.7
Cinchonidine	0.13	0.48	0.06	0.24	400.0	294	70.5
Group IV: developing solvent ethanol–acetic acid–water (60:30:10)[13]							
Morphine[d]	0.49	0.35	0.17	0.27	158.8	303	81.81
Codeine[d]	0.37	0.41	0.15	0.40	266.6	317	126.80
Heroin[e]	0.71	0.85	0.60	0.35	58.3	423	253.80
Group V: developing solvent methanol–ammonia solution (100:1.5)[13]							
Harmaline	1.58	1.61	2.54	0.38	14.9	214	81.3
Harmine	3.67	4.45	16.33	0.68	4.2	212	144.1
Group VI: developing solvent ethyl acetate–formic acid–acetic acid–water (100:11:11:27)[12]							
3hlorogenic acid	1.96	6.30	12.35	0.45	3.6	354	159.3
Caffeic acid	1.60	4.60	7.36	0.96	13.0	180	172.8
Quercetin	1.78	3.10	5.51	0.97	17.0	302	292.9
Group VII: developing solvent toluene–ethyl acetate–diethylamine (70:20:10)[12]							
Pilocarpine	0.028	–	–	0.10	–	208	20.8
Brucine[e]	1.01	1.70	1.71	0.23	13.5	466	107.1
Strychnine[e]	2.93	3.04	8.90	0.37	4.2	306	150.2
Yohimbine	0.30	0.86	0.26	0.49	188.5	354	92.0
Eserine	3.30	1.60	5.28	0.56	10.6	275	154.0
Emetine	0.36	1.16	0.41	0.56	136.5	480	268.8
Papaverine	2.61	3.50	9.13	0.62	6.8	339	210.1
Berberine	1.81	2.87	5.19	0.46	8.9	336	154.5

[a] $A = K_1 K_2$.
[b] $B = hR_F/A$.
[c] $C = MW \times R_F$.
[d] Base monohydrate.
[e] Hydrochloride salt.

(a)

R$_1$ = R$_2$ = H   Morphine

R$_1$ = H, R$_2$ = CH$_3$ Codeine

R$_1$ = R$_2$ = COCH$_3$ Heroin

(b)

R$_1$ = R$_2$ = R$_3$ = CH$_3$ Caffeine

R$_1$ = R$_2$ = CH$_3$, R$_3$ = H   Theophylline

R$_1$ = H, R$_2$ = R$_3$ = CH$_3$ Theobromine

(c)

R = OCH$_3$ (−)-Quinine   R = H   (+)-Cinchonine

R = H   (−)-Cinchonidine   R = OCH$_3$ (+)-Quinidine
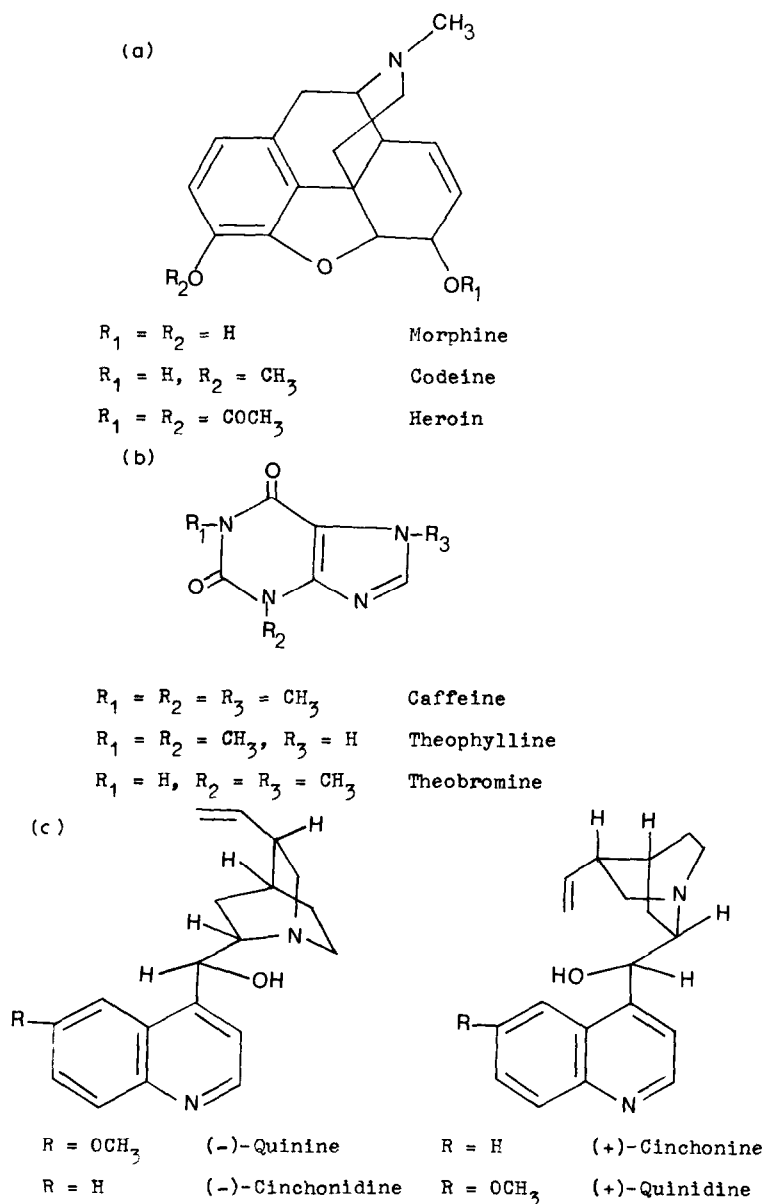
Fig. 1. Structures of compounds. (a) Phenanthrene alkaloids; (b) xanthine alkaloids; and (c) quinoline alkaloids.

chromatographic conditions. The compounds tested were detected and identified at a concentration of 0.5 μg/μl in methanol or chloroform solutions.

We tried to correlate the results obtained with the structures, and reached some interesting conclusions.

(a) Within the same group of structurally related compounds, *e.g.*, morphine, codeine and heroin[a] (Fig. 1a), there is a difference in K_1 values. The presence of a methyl group in codeine reduces its K_1 value, whereas the diacetyl group in heroin increases its K_1 value. The K_2 and C values increase in the order given.

(b) For caffeine, theobromine and theophylline (Fig. 1b), there is an increase in K_1 (0.99, 1.32 and 1.35 respectively), even though theophylline and theobromine have the same molecular mass. The K_2 values increased in the order caffeine, theophylline and theobromine (3.01, 3.76 and 3.96, respectively). Also, the B values increased in the order theobromine, theophylline and caffeine (6.88, 10.45 and 15.43, respectively), and the C values in the order theobromine, caffeine and theophylline (64.8, 89.2 and 95.4, respectively) (Table III).

(c) Within the quinoline alkaloids, which include two pairs of isomers, *viz.*, (−)-quinine, (+)-quinidine and (−)-cinchonidine, (+)-cinchonine (Fig. 1c), the presence of the methoxy group in quinine and quinidine increases the K_1 and K_2 values. Whereas the K_1 and K_2 values give no sharp differentiation between the members of each pair, the B and C values (Table III) could be regarded as a guide for differentiating between the two pairs of isomers and also between the members of each pair with the same molecular mass.

To test the reliability of identification by this method, eight unknown samples were prepared by one of the investigators and analysed by another. The eight unknowns consisted of one compound from each of the seven classes (Table III) plus a compound that was not included in this study. An initial run for each compound placed them in a particular class. From the values of absorbance ratios (K_1), the primary identification was rapidly achieved. Then, confirmation by measurement at the maximum wavelength completed the identification.

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[a] Although heroin is not a natural compound, it is included because of its structural relationship to the phenanthrene alkaloids and its legal implications.