# THE QUANTITATIVE DETERMINATION OF SOME MITRAGYNA OXINDOLE ALKALOIDS AFTER SEPARATION BY THIN-LAYER CHROMATOGRAPHY*

## PART I. ULTRAVIOLET SPECTROPHOTOMETRY

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(Received August 17th, 1967)

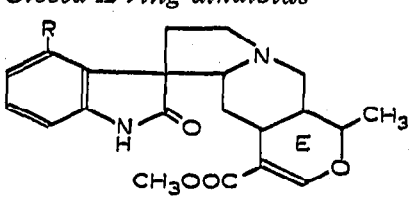
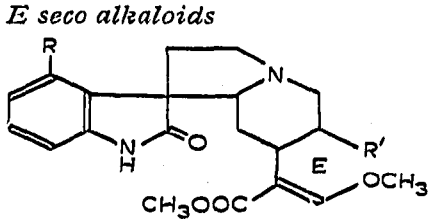
### INTRODUCTION

The quantitative determination of alkaloids by various methods, after separation by thin-layer chromatography (TLC), has been reported[1-3]. Since methods of separation and identification of the Mitragyna oxindole alkaloids by TLC have already been described [4-6] and their quantitative determination by means of ultraviolet spectrophotometry has recently been reported[7] it should be possible to determine these alkaloids in admixture, after separation by TLC provided they can be recovered quantitatively from the thin layers.

Details of the structure of the Mitragyna oxindole alkaloids are given in Table I and their ultraviolet characteristics in Table II. These indicate that the alkaloids can be divided into two groups and that the wavelengths 223 mμ and 242 mμ may

TABLE I

STRUCTURE OF MITRAGYNA OXINDOLE ALKALOIDS

| | | |
|---|---|---|
| *Closed E ring alkaloids*  | R=H | Mitraphylline<br>Isomitraphylline<br>Speciophylline<br>Uncarine F<br>Pteropodine<br>Isopteropodine |
| | $R=OCH_3$ | Javaphylline |
| *E seco alkaloids*  | $R=H, R'=CH_2CH_3$ | Rhynchophylline<br>Isorhynchophylline |
| | $R=OH, R'=CH_2CH_3$ | Rotundifoline<br>Isorotundifoline<br>Speciofoline |
| | $R=OCH_3, R'=CH_2CH_3$ | Ciliaphylline<br>Rhynchociline |

* This work forms part of a thesis submitted by M. Z. ALAM for a Ph.D. degree of the University of London.

TABLE II

ULTRAVIOLET CHARACTERISTICS OF MITRAGYNA OXINDOLE ALKALOIDS

| *Alkaloid* | *Maximum wavelengths (mμ)* | *Minimum wavelengths (mμ)* | *Shoulder wavelengths (mμ)* |
|---|---|---|---|
| *Group 1* | | | |
| Rotundifoline | 223, 290 | 275 | 206, 242 |
| Isorotundifoline | 223, 290 | 275 | 205, 242 |
| Speciofoline | 223, 290 | 274 | 204, 242 |
| *Group 2* | | | |
| Mitraphylline | 209–219, 242 | 223, 274 | |
| Isomitraphylline | 209–219, 242 | 223, 274 | |
| Speciophylline | 205–216, 242 | 223, 284 | |
| Uncarine F | 205–216, 242 | 223, 283 | |
| Pteropodine | 210–218, 245 | 223, 280 | |
| Isopteropodine | 210–218, 245 | 223, 280 | |
| Javaphylline | 209–218, 240 | 223, 291 | |
| Rhynchophylline | 210–218, 244 | 223, 274 | |
| Isorhynchophylline | 210–218, 244 | 223, 274 | |
| Ciliaphylline | 210–225, 244 | 234, 276 | |
| Rhynchophylline | 210–225, 244 | 238, 277 | |

be employed for measuring the absorbances of Group I and Group II alkaloids, respectively. Calibration curves for six alkaloids representative of these groups, previously determined by SHELLARD AND ALAM[7], are given in Fig. 1 a, b and c.

This reports gives some account of the problems relating to the quantitative determination of the oxindole alkaloids in admixture by ultraviolet spectrophotometry in association with TLC.

## METHODS

### *Thin-layer chromatography*

Glass plates 20 × 20 mm or 20 × 5 mm were covered with absorbent layer using a Desaga adjustable spreader. Adsorbents were Alumina G (Merck) and Silica Gel G (Merck); thickness of the layers was 250 mμ. The plates were activated by air drying for 15 min, followed by 1 h at 105°.

*Application of the alkaloid.* An Agla syringe containing the appropriate alkaloid in methanol, and the thin layer plates were so arranged that there was approximately 2 mm between the top of the needle and the surface of the thin layers. The plunger of the needle was turned so that it delivered a fixed volume (2.5 μl) which remained hanging as a drop. The plate was then raised so that it came into contact with the hanging drop which was then adsorbed at the starting point on the plate without damaging the surface of the thin layer and thus producing an initial spot of constant area. The solvent was evaporated by means of a gentle stream of warm air from a hair drier; further drops were then applied to the same position on the plate by the same method.

*Solvent systems*: As given in the text.

*Detection of alkaloid.* By screened U.V. light (365 mμ).

*Removal of alkaloids from layer.* Using screened U.V. light (365 mμ) a square of

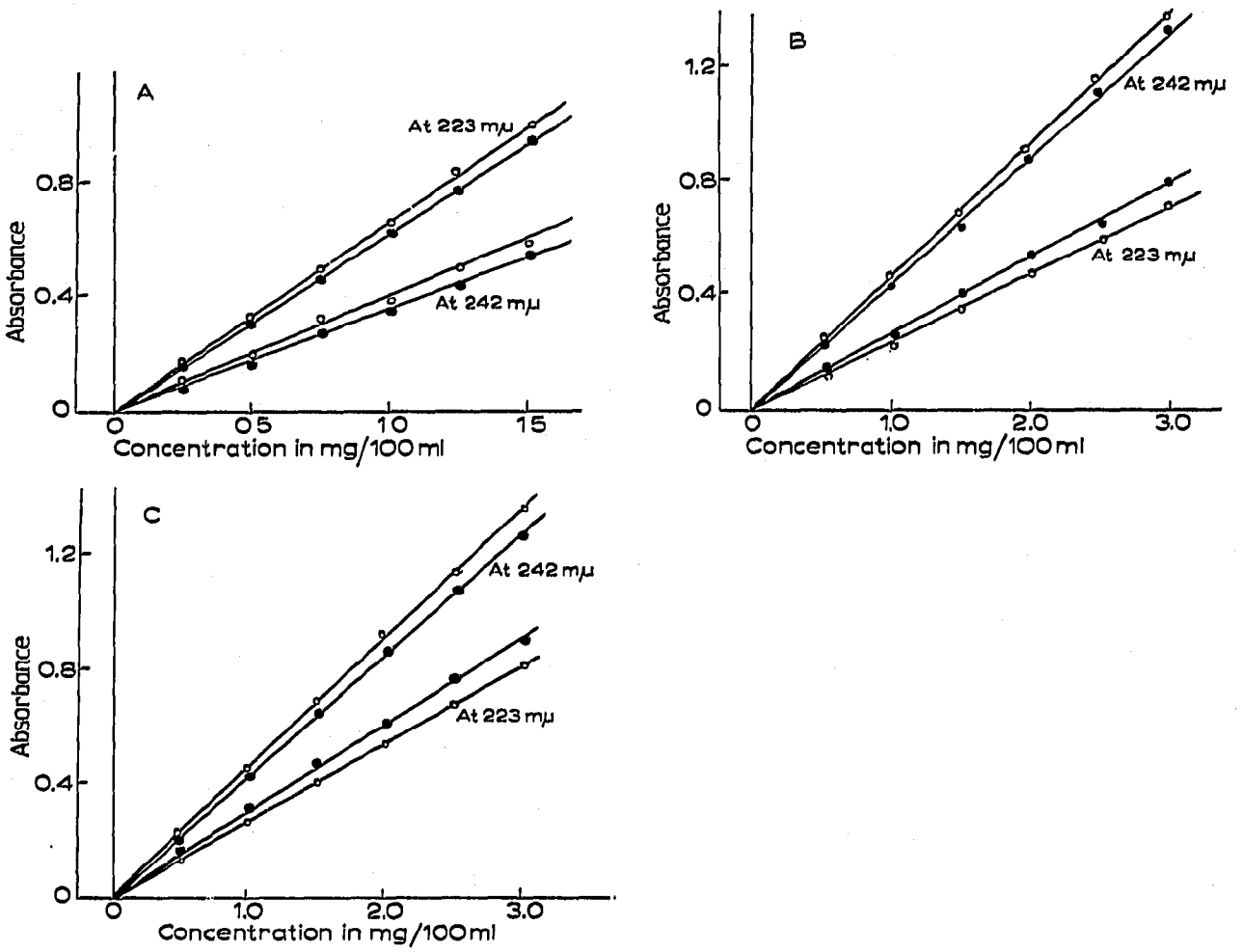



Fig. 1. (a) Calibration curves for rotundifoline (—○—○—○—) and isorotundifoline (—●—●—●—). (b) Calibration curves for rhynchophylline (—○—○—○—) and isorhynchophylline (—●—●—●—). (c) Calibration curves for mitraphylline (—○—○—○—) and isomitraphylline (—●—●—●—).

appropriate size (1 sq. in.) was marked around the alkaloid and the material from this area was then transferred by means of an aluminium foil scraper to a centrifuge tube. Elution of the alkaloids was with 5 or 10 ml methanol.

*Preparation of blank solution.* A similar area of adsorbent and at the same $R_F$ value as that where the alkaloid was removed was eluted as above.

*Spectrophotometry*

The spectrophotometers used were:

(a) a Beckman DK-2 for automatic recording;

(b) a Hilger Uvispek for manual recording.

Methanol (spectroscopically pure) was used as the solvent. The cuvettes were 1 cm quartz cells.

## RESULTS AND DISCUSSION

*Removal of the chromatographed alkaloids from the thin layers*

*Factors affecting total recovery.* The practical steps involved are (i) the removal of

the adsorbent holding the alkaloid from the plate and (ii) the elution of the alkaloid from the adsorbent. Methods previously described for the removal of the adsorbent from the plate refer to vacuum removal, the solid being retained on a sintered glass disc[8] or by means of a plug of cotton wool[5] though a more recent method involves the collection of the adsorbent in a small Soxhlet extraction thimble[9]. Application of these methods to the oxindole alkaloids gave inconsistent results, probably as a result of the elution process. In the case of the sintered glass disc and the cotton wool plug in a glass tube it is necessary to allow 3 or 4 × 5 ml quantities of solvent to percolate slowly through the collected adsorbent and it is possible that impurities are extracted from the cotton plug, though this method has the advantage that fresh plugs of cotton wool can be used each time. The sintered glass disc is frequently difficult to clean and may require the use of compressed air blown in from the reverse side. The use of the Soxhlet extraction thimble presents no problems other than the need to use large quantities of solvent which must later be removed by distillation or evaporation.

A more simple method, that of constructing a small scraper from aluminium foil and removing the adsorbent carefully commends itself since, with care, all the adsorbent can be removed from the plates and transferred to an extraction tube.

TABLE III

RECOVERY OF ALKALOIDS FROM SILICA GEL LAYERS USING DIFFERENT REMOVAL TECHNIQUES

(A) Sintered glass disc in bulb pipette. Area of layer: 1 sq. in.
(B) Cotton plug in bent glass tube. Eluting solvent: methanol (10 ml).
(C) Aluminium foil scraper.

| *Alkaloid* (80.00 μg) | *Percentage recovery* | | | *Total percentage recovery* |
|---|---|---|---|---|
| | *1st extraction* | *2nd extraction* | *3rd extraction* | |
| *Rotundifoline* | | | | |
| A | 60.25, 71.25, 41.17, 62.01, 40.80 | 30.00, 20.12, 51.20, 34.20, 45.28 | 6.25, 5.24, 3.81, nil, 9.81 | 96.50, 96.61, 96.18, 96.21, 95.89 Av. 96.27 |
| B | 60.18, 71.28, 61.91, 51.00, 70.12 | 39.21, 38.10, 25.81, 48.91, 38.10 | 1.20, nil, 2.00, 1.78, nil | 100.59, 109.38, 89.73, 101.69, 108.22 Av. 101.92 |
| C | 96.29, 98.29, 95.12, 98.05, 96.73 | nil | — | 96.29, 98.29, 95.12, 95.05, 96.73 Av. 96.30 |
| *Mitraphylline* | | | | |
| A | 50.25, 60.12, 42.00, 55.12, 41.8 | 30.81, 37.21, 31.90, 25.12, 48.12 | 16.5, nil, 23.2, 16.10, 6.12 | 97.21, 97.33, 97.02, 96.34, 95.45 Av. 96.67 |
| B | 50.25, 60.25, 40.18, 51.21, 31.81 | 45.20, 21.20, 48.00, 48.12, 50.18 | 6.21, 7.18, 7.91, 3.90, 7.19 | 101.56, 88.65, 96.09, 103.23, 89.18 Av. 95.75 |
| C | 98.21, 97.29, 97.21, 98.01, 97.85 | nil | — | 91.21, 97.29, 97.21, 98.01, 97.85 Av. 97.71 |

No additional impurities are added and there is no difficulty in cleaning the apparatus. Only small quantities of solvent are needed for eluting the alkaloid by shaking the adsorbent with 5 ml solvent in the tube and centrifuging. Table III shows a comparison of the recovery of two alkaloids using the different methods of removal of the adsorbent from the plate.

The actual recovery of the alkaloid from the adsorbent involves (i) method of elution and (ii) choice of solvent. The method of elution will depend upon the technique used for removal of the adsorbent from the layer and is of no great significance except with regard to the total volume of solvent required for complete elution.

With regard to the choice of solvent, many different solvents have been used previously though no reasons have been given for the particular choice of solvent. If no attention need be paid to the possibility of extracting impurities from the adsorbent itself (see later) it would be preferable to use the more polar solvents, *e.g.*, methanol. However, in order to minimise the risk of extracting impurities from the adsorbent an attempt was made to elute the alkaloids with other solvents. The two alkaloids selected for the test were rotundifoline, which, on silica gel layers with ethyl acetate, ethanol or methanol as the solvent, moves further than the other

TABLE IV

RECOVERY OF ALKALOIDS FROM THIN LAYERS OF DIFFERENT ABSORBENTS

Solvent system: chloroform.
Removal technique: aluminium scraper.
Eluting solvent: methanol (10 ml).
Area of layer taken (for sample and reference): 1 sq. in.

| *Alkaloid* (*80.00 μg*) | *Adsorbent* | *Percentage recovery* | | *Total percentage recovery* |
|---|---|---|---|---|
| | | *1st extraction* | *2nd extraction* | |
| Rotundifoline | Alumina | 97.25, 96.21, 97.12, 95.95, 97.21 | nil | 97.25, 96.21, 97.12, 95.95, 97.21 Av. 96.74 |
| | Magnesium oxide | 95.21, 96.12, 97.12, 98.00, 98.00 | nil | 95.21, 96.12, 97.12, 98.00, 98.00 Av. 96.89 |
| | Magnesium silicate | 97.21, 95.20, 95.28, 97.21, 95.29 | nil | Av. 96.03 |
| | Silica gel | see Table III | see Table III | Av. 96.30 |
| Mitraphylline | Alumina | 95.82, 96.92, 95.81, 97.91, 97.98 | nil | 95.82, 96.92, 95.81, 97.91, 97.98 Av. 96.88 |
| | Magnesium oxide | 95.21, 95.12, 94.12, 96.01, 97.10 | 0.95, 1.01, 0.18, nil, nil | 96.16, 96.13, 94.30, 96.01, 97.10 Av. 95.94 |
| | Magnesium silicate | 95.12, 95.81, 95.12, 97.12, 98.12 | nil, 1.21, 0.81, 0.88, nil | 95.12, 96.02, 95.93, 98.00, 98.12 Av. 96.63 |
| | Silica gel | see Table III | see Table III | Av. 97.71 |

oxindole alkaloids, and mitraphylline, which, under the same conditions moves less than the other alkaloids.

In thin-layer chromatography, both adsorption and partition chromatography may be involved so that the solvent system which gives the highest $R_F$ values does not necessarily indicate that it is the most suitable for eluting purposes. It is more likely, however, that the most suitable solvents will be those which are highly polar and this was confirmed by results obtained, since methanol is to be preferred as the solvent for eluting the oxindole alkaloids, similar results being obtained when the adsorbent is alumina, magnesium oxide and magnesium silicate (Tble IV). Except when methanol was used as solvent it was not possible to recover completely the slowest moving alkaloid—mitraphylline—with one extraction. The question thus arose as to whether rotundifoline could be quantitatively recovered from the adsorbent when the solvent system used for developing the plate was such that this alkaloid too, had a low $R_F$ value. Solvent systems were selected which, with silica gel, give $R_F$ values for rotundifoline from 0.1 to 0.8 but as indicated in Table V there is good recovery in each case.

Elution on a quantitative basis is possible with all six oxindole alkaloids and this is irrespective of their $R_F$ values or the solvent system and adsorbent layer used for the purpose of their separation.

TABLE V

RECOVERY OF ROTUNDIFOLINE FROM THIN LAYER ADSORBENT AFTER DEVELOPING WITH DIFFERENT SYSTEMS

Load: 80.0 μg.
Removal technique: aluminium foil scraper.
Eluting solvent: methanol (10 ml).

| *Chromatographic system* | *$R_F$ values* | *Range and average of recovery (%)* | *% Coefficient of variation* |
|---|---|---|---|
| Silica gel/chloroform | 0.09–0.12 | 94.43–96.57–98.74 | 3.02 |
| Silica gel/ether | 0.30–0.35 | 95.13–97.22–98.62 | 1.52 |
| Silica gel/ether–diethylamine (99:1) | 0.40–0.50 | 93.50–97.20–99.87 | 5.52 |
| Silica gel/chloroform–acetone (5:4) | 0.66–0.77 | 95.24–97.35–100.18 | 3.15 |
| Silica gel/methanol | 0.72–0.82 | 94.35–96.60–98.24 | 1.72 |
| Alumina/chloroform–cyclohexane (7:3) | 0.27–0.32 | 94.16–96.59–99.38 | 1.27 |
| Alumina/chloroform | 0.50–0.54 | 95.26–97.12–98.14 | 1.02 |

TABLE VI

ABSORBANCE OF REFERENCE SOLUTION FROM SILICA GEL LAYERS (UNDEVELOPED PLATES)

Removal technique: aluminium foil scraper.
Elution solvent: methanol (10 ml).

| *Area of thin layer (sq. in)* | *Range and average absorbance at 223 mμ* | *% Coefficient of variation* | *Range and average absorbance at 242 mμ* | *% Coefficient of variation* |
|---|---|---|---|---|
| 1.00 | 0.02–0.04–0.050 | 32.78 | 0.01–0.012–0.19 | 31.81 |
| 2.25 | 0.079–0.094–0.105 | 12.04 | 0.029–0.037–0.050 | 23.09 |
| 4.0 | 0.115–0.145–0.160 | 12.89 | 0.038–0.051–0.062 | 22.46 |
| 6.25 | 0.190–0.221–0.260 | 12.83 | 0.065–0.073–0.085 | 11.12 |
| 9.0 | 0.249–0.282–0.310 | 8.69 | 0.075–0.087–0.093 | 8.45 |

*Factors relating to the extraction of impurities from the adsorbent.* An ideal solvent for eluting the alkaloids would be one which would extract the alkaloid quantitatively but would not extract any of the impurities present in the adsorbent which are likely to interfere with the estimation based on the ultraviolet characteristic of the alkaloid. The fact that methanol is the most suitable solvent for eluting all the six oxindole alkaloids means that it is not sufficiently selective and is likely therefore to extract impurities from the adsorbent. The problem of eliminating or controlling the effect of impurities has been investigated by previous workers along two lines: (i) by treating the adsorbent prior to spreading the plates[1], and (ii) by treating the adsorbent layer on the plate prior to use[1,10]. The evidence of those who attempted to eliminate the interfering substances by extraction of the adsorbent prior to spreading the plates suggests that this is not a very suitable method.

Some attempts were made, however, to eliminate the interfering substances by pretreating the prepared layers prior to use. It was necessary first, to ascertain the actual absorbance of the impurities extracted from undeveloped plates and the results in Table VI show the average absorbance of different areas of silica gel layers at 223 mμ and 242 mμ. They indicate a wide variation in the absorbance of fixed areas of layer from different plates and it is obvious that methanol extracts a considerable amount of impurities which give significant absorbance both at 223 mμ and 242 mμ.

Table VII shows the absorbance of a fixed area of silica gel layers extracted from plates of different batches of adsorbent after development with chloroform–acetone (5:4), and this wide variation in the absorbance from the different plates rules out the possibility of the use of average absorbance figures at either wavelength as a standard figure for all determinations.

Table VIII shows the variation of absorbance of a fixed area of the silica gel extracted from different positions (equivalent to the $R_F$ values of the alkaloids) on the plates. The results show that both at 223 mμ and 242 mμ the average absorbances are higher when the layer near the solvent front is extracted than when the layer near the starting point is extracted. This probably arises from the fact that as the solvent moves along the thin layer in the development tank it takes with it some of the soluble impurities so that the portion of the thin layer plate near the starting point, having been washed with more solvent, contains less impurities than the portion near the solvent front.

The significant difference in the absorbance of the blank solution extracted from the different parts of the same plate shows that the solution extracted from a

TABLE VII

ABSORBANCE OF REFERENCE SOLUTION FROM DIFFERENT SILICA GEL LAYERS, ALL TAKEN AT $R_F$ 0.50

Solvent system: chloroform–acetone (5:4).
Area of adsorbent taken: 1.0 sq.in.
Eluting solvent: methanol (10 ml).

| *Wavelength (mμ)* | *Absorbance* | *Average* | *% Coefficient of variation* |
|---|---|---|---|
| 223 | 0.039, 0.027, 0.040, 0.019, 0.031, 0.029, 0.025, 0.031, 0.045, 0.029 | 0.034 | 28.34 |
| 242 | 0.021, 0.012, 0.022, 0.013, 0.019, 0.012, 0.015, 0.022, 0.019, 0.025 | 0.018 | 26.65 |

TABLE VIII

ABSORBANCE OF REFERENCE SOLUTIONS FROM DIFFERENT PARTS OF THE SAME SILICA GEL LAYER, USING DIFFERENT SOLVENT SYSTEMS

Area of layer: 1 sq.in.
Eluting solvent: methanol (10 ml).

| *Solvent system* | *$R_F$ value of portion taken* | *Range and average absorbance at 223 mμ* | *Range and average absorbance at 242 mμ* |
|---|---|---|---|
| Chloroform–acetone (5:4) | 0.05 | 0.021–0.0312–0.040 | 0.009–0.0118–0.015 |
| | 0.30 | 0.028–0.0378–0.050 | 0.015–0.0176–0.022 |
| | 0.50 | 0.039–0.0430–0.048 | 0.018–0.0192–0.021 |
| | 0.95 | 0.075–0.0978–0.120 | 0.035–0.0536–0.062 |
| Ether | 0.05 | 0.020–0.0264–0.035 | 0.008–0.0140–0.015 |
| | 0.30 | 0.033–0.036–0.042 | 0.009–0.0130–0.017 |
| | 0.50 | 0.042–0.0476–0.072 | 0.012–0.0136–0.020 |
| | 0.95 | 0.055–0.0674–0.080 | 0.015–0.0166–0.020 |

TABLE IX

ABSORBANCE OF REFERENCE SOLUTION FROM SILICA GEL LAYERS AFTER DEVELOPMENT WITH DIFFERENT SOLVENT SYSTEMS

Area of adsorbent taken: 1 sq.in. ($R_F$ 0.5).
Eluting solvent: methanol (10 ml).
(A) Untreated plates; (B) pretreated by prior development with the solvent system.

| *Solvent system* | | *Absorbance* | |
|---|---|---|---|
| | | *223 mμ* | *242 mμ* |
| Chloroform–acetone (5:4) | A | 0.069; 0.062; 0.051; 0.042; 0.035; Av. 0.046 | 0.020; 0.015; 0.020; 0.030; 0.035; Av. 0.024 |
| | B | 0.079; 0.089; 0.098; 0.082; 0.072; Av. 0.084 | 0.025; 0.014; 0.022; 0.040; 0.019; Av. 0.024 |
| Ether | A | 0.050; 0.055; 0.058; 0.068; 0.072; Av. 0.061 | 0.012; 0.008; 0.009; 0.007; 0.015; Av. 0.010 |
| | B | 0.045; 0.065; 0.055; 0.035; 0.032; Av. 0.047 | 0.015; 0.010; 0.007; 0.009; 0.012; Av. 0.011 |
| Chloroform | A | 0.045; 0.052; 0.055; 0.062; 0.068; Av. 0.056 | 0.011; 0.008; 0.009; 0.015; 0.012; Av. 0.009 |
| | B | 0.052; 0.056; 0.045; 0.066; 0.072; Av. 0.058 | 0.015; 0.010; 0.012; 0.009; 0.008; Av. 0.011 |
| Methanol | A | 0.025; 0.035; 0.047; 0.050; 0.057; Av. 0.043 | 0.015; 0.018; 0.008; 0.009; 0.013; Av. 0.013 |
| | B | 0.022; 0.035; 0.027; 0.035; 0.038; Av. 0.031 | 0.012; 0.015; 0.012; 0.088; 0.012; Av. 0.010 |

particular portion of the layer can only be used as a blank for an alkaloid of similar $R_F$ value otherwise a significant error would be introduced. The results obtained, show this to be true where, in the case of rhynchophylline, a variation of 92.42 to 105.2 % is observed.

The fact that impurities move to the solvent front by development suggested the possibility of eliminating interfering substances by preliminary development of the layer by suitable solvents. Table IX shows the results of washing the adsorbent layers by a preliminary development with solvent systems of varying polarities. The absorbance readings do not however show any significant reduction of impurities; even methanol, the most polar solvent failing to show any marked reduction. Surprisingly when acetone–chloroform (4:5) is used as the washing solvent there is a marked increase in absorbance at 223 m$\mu$ which may be due to the fact that a trace of acetone, which has a high absorbance in the ultraviolet region, remains adsorbed by the layer.

Hence an attempt to standardise the absorbance of the blank and the attempt to reduce extractable impurities from the layer did not provide a satisfactory solution to the elimination of the interfering substances from the adsorbent layer and it is concluded that the elimination of the interfering substances can best be obtained by extracting a similar area of adsorbent from the same plate and removed at a position of identical $R_F$ value to the alkaloid under examination, this solution being used as the reference solution.

*Estimation of alkaloids in admixture*

Tables X and XI show results obtained with mixtures of 2 and 3 alkaloids which can be separated sufficiently on the thin layer. The results show that there is no interference between the alkaloids in admixture, that no isomerisation of the alkaloids occurs and that the separation of the mixed alkaloids is quantitative. However, it is not always possible to separate the alkaloids sufficiently to enable them to be removed from the layer as individual alkaloids and in such cases it may be pos-

TABLE X

ESTIMATION OF ALKALOIDS IN TWO COMPONENT MIXTURES
(a) Rotundifoline and isorotundifoline.
(b) Mitraphylline and isomitraphylline.
Solvent system: Silica gel/chloroform–acetone (5:4).

| *Alkaloid* | *Amount added* ($\mu$g) | *Amount found* ($\mu$g) | *Average* ($\mu$g) | *% Coefficient of variation* |
|---|---|---|---|---|
| (a) Isorotundifoline | 10.0 | 9.51, 9.91, 9.81, 9.01, 9.19 | 9.48 | 4.07 |
| $R_F$ = 0.40–0.47 | 20.0 | 18.52, 18.25, 19.29, 19.75, 18.09 | 18.80 | 3.84 |
| | 30.0 | 27.54, 28.54, 28.59, 29.59, 27.69 | 28.38 | 2.96 |
| Rotundifoline | 18.0 | 18.45, 19.05, 19.15, 19.75, 19.25 | 19.18 | 2.60 |
| $R_F$ = 0.66–0.74 | 36.0 | 37.25, 37.75, 38.09, 39.05, 37.15 | 2.06 | |
| | 54.0 | 54.23, 56.14, 56.10, 54.13, 54.50 | 55.00 | 1.85 |
| (b) Mitraphylline | 18.0 | 18.04, 18.90, 17.94, 18.52, 19.20 | 18.52 | 2.91 |
| $R_F$ = 0.38–0.43 | 36.0 | 36.52, 36.55, 36.75, 36.78, 36.01 | 36.52 | 0.87 |
| | 54.0 | 51.25, 52.52, 53.25, 52.59, 52.59 | 52.44 | 1.44 |
| | 72.0 | 74.52, 71.44, 72.54, 72.65, 72.59 | 72.71 | 1.39 |
| Isomitraphylline | 7.0 | 6.52, 6.89, 7.00, 7.25, 6.92 | 6.89 | 3.81 |
| $R_F$ = 0.60–0.67 | 14.0 | 13.15, 13.25, 13.19, 13.95, 14.50 | 13.60 | 4.35 |
| | 21.0 | 21.05, 20.75, 21.95, 22.00, 21.75 | 21.50 | 2.58 |
| | 28.0 | 27.29, 28.25, 28.45, 28.85, 27.35 | 28.05 | 2.45 |

TABLE XI

ESTIMATION OF ALKALOIDS IN A THREE COMPONENT MIXTURE
System: Silica gel/chloroform–acetone (5:4).

| *Alkaloids and amount added* | *Amount found (μg)* | *Average (μg)* | *% Coefficient of variation* |
|---|---|---|---|
| Rotundifoline (20.60 μg) $R_F$ = 0.06–0.74 | 19.21, 20.51, 19.52, 19.05, 19.00 | 19.46 | 3.11 |
| Mitraphylline (17.60 μg) $R_F$ = 0.38–0.43 | 17.21, 17.42, 17.25, 17.50, 17.20 | 17.32 | 0.78 |
| Rhynchophylline (8.00 μg) $R_F$ = 0.24–0.33 | 7.90, 7.65, 7.55, 7.49, 7.72 | 7.66 | 2.08 |

sible to remove adjacent alkaloids in pairs and to estimate them in a binary mixture by taking spectrophotometric readings at two wavelengths (223 mμ and 242 mμ are suitable, SHELLARD AND ALAM[7]) and applying the simultaneous equations:

$$A_{223} = C^{I}K_{223} + C^{II}K_{223}{}^{II}$$
$$A_{242} = C^{I}K_{242} + C^{II}K_{242}{}^{II}$$

where $A$, $C$ and $K$ are absorbance, concentration in mg/ml and specific extinction coefficient respectively, I and II represent the two alkaloids which must be in different groups.

The specific extinction coefficients for the six oxindole alkaloids (using methanol as solvent) are given in Table XII.

Table XIII shows the results obtained for mitraphylline and isorotundifoline when they are estimated individually and when they are estimated in admixture. It can be seen that there is no significant difference between the results obtained in each case and this is substantiated when a mixture of four alkaloids is chromatographed on silica gel with chloroform–acetone (5:4) to yield two spots, one containing rotundifoline and isorhynchophylline and the other isorotundifoline and mitraphylline.

Attempts to separate 5 and 6 component mixtures into groups containing not more than two alkaloids were not always successful and could only be achieved if the amounts of the alkaloid were very small. Thus the method of analysis just described is not altogether applicable, and it was considered necessary to consider other procedures.

Different separation patterns are achieved with the same group of alkaloids

TABLE XII

SPECIFIC EXTINCTION COEFFICIENTS OF THE OXINDOLE ALKALOIDS (IN METHANOL)

| | *Group I* | | *Group II* | | | |
|---|---|---|---|---|---|---|
| | *Rotundifoline* | *Isorotundifoline* | *Rhynchophylline* | *Isorhynchophylline* | *Mitraphylline* | *Isomitraphylline* |
| 223 mμ | 66.05 | 64.05 | 23.62 | 24.97 | 26.60 | 30.02 |
| 242 mμ | 40.00 | 36.63 | 44.94 | 43.94 | 45.51 | 42.98 |

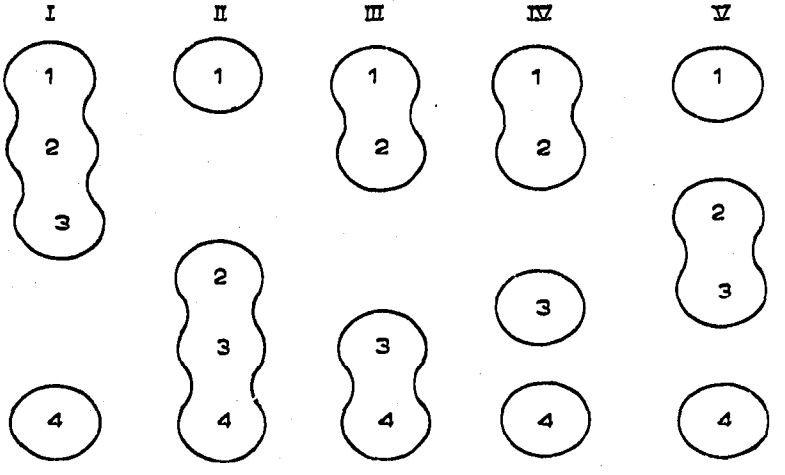
TABLE XIII

ESTIMATION OF MITRAPHYLLINE AND ISOROTUNDIFOLINE IN ADMIXTURE
(a) As individual spots; system: alumina/chloroform.
(b) As single spot; system: silica gel/chloroform–acetone (5:4).

| *Alkaloids and amount added* | *Amount found (μg)* | *Average (μg)* | *% Coefficient of variation* |
|---|---|---|---|
| (a) Mitraphylline (31.20 μg) $R_F$ = 0.15–0.20 | 29.69, 29.47, 30.25, 29.25, 31.15 | 30.00 | 2.56 |
| Isorotundifoline (41.20 μg) $R_F$ = 0.42–0.44 | 40.69, 39.25, 39.07, 41.25, 40.15 | 40.12 | 2.31 |
| (b) Mitraphylline (31.20 μg) $R_F$ = 0.38–0.44 | 28.75, 30.79, 30.25, 31.15, 30.15 | 30.26 | 3.02 |
| Isorotundifoline (41.20 μg) $R_F$ = 0.38–0.44 | 38.75, 38.29, 39.15, 40.02, 40.17 | 39.50 | 1.49 |

when using different thin layer systems and it was thought that use could be made of this for differential spectrophotometry.

If consideration is given, for example, to the separation of four alkaloids by five different systems so that different patterns are obtained but without all the alkaloids ever being sufficiently well separated for individual elution, such separations can be represented as follows:




Alkaloid 2 never occurs as a completely separated alkaloid in any system. Only 1, 3 and 4 can be estimated as individuals alkaloids. 1 and 2, 3 and 4, and 2 and 3 can be estimated as two component mixtures provided they are not in the same group as far as their absorption characteristics are concerned. But if alkaloids 1, 2 and 3 are in the same group it is not possible to estimate alkaloid 2 directly.

However, by using the eluted alkaloid 1 (from systems II and V) in the reference cell and the elution of the mixture of alkaloids 1 and 2 in the test cell (from either systems III or IV), provided account can be taken of the difference in the background absorbance of the two solutions, due to the elutions being made from different plates, of different areas of adsorbent and possibly at different $R_F$ values, the

absorbance of alkaloid 2 can be read directly using the principle of differential spectrophotometry.

Alternatively, using systems III and V the alkaloids could be estimated as follows:

Alkaloid 1 from system V directly;

Alkaloid 2 by subtracting the absorbance of alkaloid 1 (system V) from the absorbance of 1 and 2 (system III);

Alkaloid 3 by subtracting the absorbance of alkaloid 4 (system V) from the absorbance of 3 and 4 (system III);

Alkaloid 4 from system V directly.

This is simply a question of arithmetic and requires no additional practical manipulation. Attempts to apply this to a mixture of the six oxindole alkaloids, however, presented some difficulties since actual separation of the alkaloids into pairs was not always achieved. Fig. 2 shows the actual chromatograms obtained with mixtures of the six alkaloids using four different systems.

It is necessary to consider the effect of the spot containing 3 or 4 alkaloids which cannot be further separated. Differential spectrophotometry can be applied provided that the eluate obtained from the blank adsorbent is the same in each cell. Thus the adsorbent containing one group of alkaloids is placed in one centrifuge tube and the same area of the blank adsorbent of the same $R_F$ value obtained from the same plate is placed in the second centrifuge tube as usual. The adsorbent containing the other group of alkaloids is placed in the second centrifuge tube and the same area of the blank adsorbent of the same $R_F$ value obtained from the second plate is placed in the first centrifuge tube. Both are extracted with the same volume of methanol

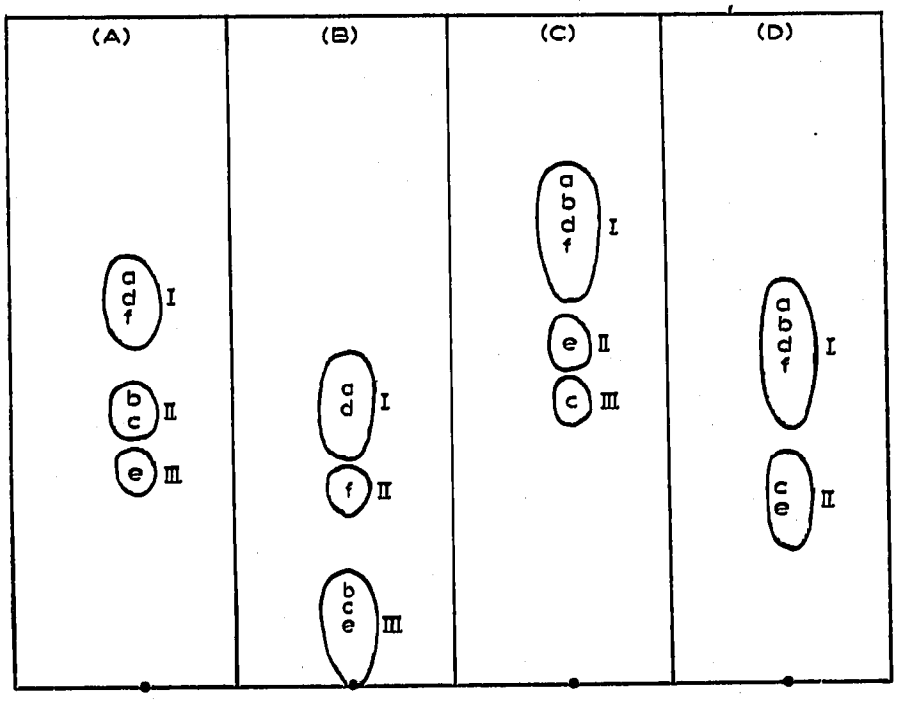



Fig. 2. Chromatogram of a mixture of the six oxindole alkaloids. a = Rotundifoline; b = isorotundifoline; c = rhynchophylline; d = isorhynchophylline; e = mitraphylline; f = isomitraphylline. Chromatographic systems: (A) Silica gel/chloroform–acetone (5:4); (B) silica gel/ether; (C) alumina/chloroform; (D) alumina/chloroform–cyclohexane (7:3).

and after centrifuging, the supernatant liquid in the two cells is examined, the actual absorbance measured being due entirely to the alkaloid(s) present in one spot but not in the other. If only one alkaloid is involved the measurement of absorbance can be related directly to the calibration curve for the alkaloid. If two alkaloids of different groups are involved the absorbance is measured at 223 mμ and 242 mμ. The method can also be applied if the alkaloids involved are rotundifoline and isorotundifoline (SHELLARD AND ALAM[7]).

TABLE XIV

ESTIMATION OF THE ALKALOIDS IN A SIX-COMPONENT MIXTURE (see Fig. 2)

Systems: (A) Silica gel/chloroform–acetone (5:4),
(B) Silica gel/ether,
(C) Alumina/chloroform,
(D) Alumina/chloroform–cyclohexane (7:3).

*1. Using adsorbent eluate as reference solution*

(*a*) *As individual alkaloids*

| *Alkaloid and amount* | *Solvent system* | *Spot* | *Amount found (μg)* | *Average (μg)* | *% Coefficient of variation* |
|---|---|---|---|---|---|
| Rhynchophylline (33.00 μg) | A | III | 33.45, 31.44, 31.67, 33.32, 31.22 | 32.22 | 3.29 |
| | C | III | 33.23, 31.22, 31.44, 31.00, 32.54 | 31.82 | 2.88 |
| Mitraphylline (30.20 μg) | C | II | 33.00, 32.11, 33.90, 31.67, 34.57 | 33.06 | 3.65 |
| Isomitraphylline (34.00 μg) | B | II | 35.19, 33.82, 32.92, 33.37, 35.01 | 34.06 | 2.95 |

(*b*) *As binary mixture*

| *Alkaloid and amount* | *Solvent system* | *Spot* | *Amount found (μg)* | *Average (μg)* | *% Coefficient of variation* |
|---|---|---|---|---|---|
| Rotundifoline (62.00 μg) | B | I | 65.78, 63.28, 63.60, 65.61, 63.19 | 64.30 | 2.00 |
| Isorhynchophylline (33.50 μg) | | | 36.18, 35.57, 36.01, 34.89, 35.45 | 35.64 | 1.43 |
| Isorotundifoline (36.50 μg) | A | II | 37.50, 35.61, 37.97, 36.50, 35.59 | 36.64 | 2.97 |
| Mitraphylline (30.20 μg) | | | 29.71, 30.53, 28.35, 31.60, 29.95 | 30.04 | 3.88 |

(*continued on p. 485*)

TABLE XIV (*continued*)

*2. By arithmetical method*

(*a*) *As individual spot*

| *Alkaloid and amount* | *Adsorbance of sample spot eluate* | | *Absorbance to be subtracted* | | *Resultant absorbance* | | *Amount found (μg)* | *Average (μg)* | *% Coefficient of variation* |
|---|---|---|---|---|---|---|---|---|---|
| | *223 mμ* | *242 mμ* | *223 mμ* | *242 mμ* | *223 mμ* | *242 mμ* | | | |
| Mitraphylline (30.20 μg) | | 0.288 | | 0.144 | | 0.144 | 31.53 | 30.74 | 5.62 |
| | | 0.278 | | (AIII) | | 0.134 | 29.34 | | |
| | | 0.292 | | | | 0.148 | 32.41 | | |
| | | 0.274 | | | | 0.130 | 28.47 | | |
| | | 0.290 | | | | 0.146 | 31.97 | | |
| | | (DII) | | | | | | | |
| Rhynchophylline (33.00 μg) | | 0.416 | | 0.273 | | 0.143 | 31.89 | 33.92 | 7.26 |
| | | 0.427 | | (AII) | | 0.154 | 34.34 | | |
| | | 0.440 | | | | 0.167 | 37.24 | | |
| | | 0.430 | | | | 0.157 | 35.01 | | |
| | | 0.413 | | | | 0.140 | 31.22 | | |
| | | (BIII) | | | | | | | |
| Isomitraphylline (34.00 μg) | 0.605 | 0.545 | 0.486 | 0.392 | 0.119 | 0.153 | 34.73 | 34.12 | 1.14 |
| | 0.600 | 0.542 | (BI) | | 0.114 | 0.150 | 34.05 | | |
| | 0.607 | 0.540 | | | 0.121 | 0.148 | 33.60 | | |
| | 0.610 | 0.542 | | | 0.124 | 0.150 | 34.05 | | |
| | 0.602 | 0.542 | | | 0.116 | 0.150 | 34.05 | | |
| | (AI) | | | | (b) | | | | |
| Isorotundifoline (36.50 μg) | 0.835 | 0.690 | 0.605 | 0.542 | 0.230 | 0.148 | 35.88 | 34.82 | 4.91 |
| | 0.840 | 0.699 | (AI) | | 0.235 | 0.157 | 36.66 | | |
| | 0.820 | 0.692 | | | 0.215 | 0.150 | 33.54 | 34.82 | |
| | 0.814 | 0.685 | | | 0.209 | 0.143 | 32.60 | | |
| | 0.832 | 0.687 | | | 0.227 | 0.145 | 35.41 | | |
| | (CI) | | | | (a) | | | | |
| Isorotundifoline (36.50 μg) | 0.834 | 0.685 | 0.605 | 0.542 | 0.229 | 0.143 | 35.72 | 35.48 | 3.67 |
| | 0.819 | 0.678 | (AI) | | 0.214 | 0.136 | 33.38 | | |
| | 0.842 | 0.682 | | | 0.237 | 0.140 | 36.97 | | |
| | 0.832 | 0.687 | | | 0.227 | 0.145 | 35.41 | | |
| | 0.835 | 0.684 | | | 0.230 | 0.142 | 35.88 | | |
| | (DI) | | | | | | | | |

(*b*) *As binary mixture*

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Rotundifoline (62.00 μg) | 0.835 | 0.690 | 0.340 | 0.298 | 0.495 | 0.392 | 67.02, 67.01 | 65.32 | 3.52 |
| | 0.840 | 0.699 | (a + b) | | 0.500 | 0.401 | 63.01, 62.60, | | |
| | 0.820 | 0.692 | | | 0.480 | 0.394 | 66.97 | | |
| Isorhynchophylline (33.50 μg) | 0.814 | 0.685 | | | 0.474 | 0.387 | 32.91, 35.03, | 34.62 | 3.46 |
| | 0.832 | 0.687 | | | 0.492 | 0.389 | 36.96, 35.74, | | |
| | (CI) | | | | | | 32.50 | | |
| Rotundifoline (62.00 μg) | 0.834 | 0.685 | 0.340 | 0.298 | 0.494 | 0.387 | 67.46, 64.63, | 65.86 | 4.31 |
| | 0.819 | 0.679 | (a + b) | | 0.479 | 0.381 | 62.65, 66.70, | | |
| | 0.812 | 0.681 | | | 0.472 | 0.383 | 67.94 | | |
| Isorhynchophylline (33.50 μg) | 0.832 | 0.687 | | | 0.492 | 0.389 | 31.33, 32.46, | 32.22 | 5.50 |
| | 0.835 | 0.684 | | | 0.495 | 0.386 | 34.77, 32.49 | | |
| | (DI) | | | | | | | | |

(*continued on p. 486*)

TABLE XIV (*continued*)

*3. By differential spectrophotometry*

(*a*) *As individual alkaloids*

| *Alkaloid and amount* | *Spot as sample solution* | *Spot as reference solution* | *Differential absorbance* | | *Amount found (μg)* | *Average (μg)* | *% Coefficient of variation* |
|---|---|---|---|---|---|---|---|
| | | | *223 mμ* | *242 mμ* | | | |
| Isorotundifoline (36.50 μg) | AII | CII | 0.252<br>0.250<br>0.249 | 0.242<br>0.245 | 39.31, 37.75, 39.00, 38.22, 38.84 | 38.62 | 1.57 |
| | BIII | DII | 0.241<br>0.251<br>0.245 | 0.251<br>0.242 | 37.59, 39.16, 39.16, 37.75, 38.22 | 38.40 | 1.87 |
| | CI | AI | 0.248<br>0.247<br>0.246 | 0.242<br>0.250 | 38.62, 37.75, 38.53, 39.00, 38.57 | 38.42 | 1.33 |
| | DI | AI | 0.247<br>0.239<br>0.238 | 0.247<br>0.239 | 38.53, 38.53, 37.20, 37.28, 37.12 | 37.74 | 1.86 |
| Rhynchophylline (33.00 μg) | BIII | AII | 0.148<br>0.150<br>0.144 | 0.150<br>0.143 | 33.00, 33.45, 33.45, 31.88, 32.11 | 32.80 | 2.32 |
| | DII | CII | 0.151<br>0.143<br>0.152 | 0.142<br>0.140 | 33.67, 31.66, 31.89, 31.22, 33.89 | 32.48 | 3.79 |
| Mitraphylline (30.20 μg) | DII | CIII | 0.149<br>0.151<br>0.142 | 0.142<br>0.151 | 32.63, 31.10, 33.07, 33.07, 31.10 | 32.20 | 3.18 |
| Isomitraphylline (34.00 μg) | AI | BI | 0.158<br>0.148<br>0.142 | 0.145<br>0.152 | 35.87, 32.92, 33.60, 34.50, 32.21 | 33.80 | 4.15 |
| (*b*) *As binary mixture* | | | | | | | |
| Rotundifoline (62.00 μg) | AI | BII | 0.492<br>0.485 | 0.402<br>0.395 | 64.91, 64.17, 65.36, 63.81, 63.92 | 64.40 | 1.06 |
| Isorhynchophylline (33.50 μg) | | | 0.491<br>0.482<br>0.485 | 0.397<br>0.392<br>0.393 | 37.21, 36.21, 35.60, 35.60, 35.82 | 36.08 | 1.86 |
| Isorotundifoline (36.50 μg) | BIII | AIII | 0.335<br>0.329 | 0.271<br>0.270 | 36.37, 35.14, 35.97, 35.02, 34.95 | 35.50 | 1.85 |
| Mitraphylline (30.20 μg) | | | 0.335<br>0.332 | 0.275<br>0.276 | 29.65, 28.05, 31.18, 32.19, 30.01 | 30.24 | 5.15 |

TABLE XV

COMPARISON OF RESULTS BY THE THREE METHODS

| *Alkaloid* | *Theoretical amount (μg)* | *Method I* | | *Method II* | | *Method III* | |
|---|---|---|---|---|---|---|---|
| | | *Amount found (μg)* | *% Coefficient of variation* | *Amount found (μg)* | *% Coefficient of variation* | *Amount found (μg)* | *% Coefficient of variation* |
| Rotundifoline | 62.00 | 64.30 | 2.00 | 65.59 | 3.91 | 64.40 | 1.06 |
| Isorotundifoline | 36.50 | 36.64 | 2.97 | 35.15 | 4.29 | 38.48 | 1.65 |
| Rhynchophylline | 33.00 | 32.02 | 3.08 | 33.92 | 7.26 | 32.64 | 3.08 |
| Isorhynchophylline | 33.50 | 35.64 | 1.43 | 33.42 | 4.86 | 36.08 | 1.86 |
| Mitraphylline | 30.20 | 33.06 | 3.65 | 30.74 | 5.62 | 31.22 | 4.16 |
| Isomitraphylline | 34.00 | 34.06 | 2.95 | 34.12 | 1.14 | 33.80 | 4.15 |

Consideration of the estimation of each of the six alkaloids in admixture will illustrate this method.

*Rotundifoline.* In association with isorhynchophylline.

1 BI, using blank silica gel eluate as reference solution.

2 AI, using BII as reference solution.

3 By subtracting the absorbance of isorotundifoline and isomitraphylline (a + b) from the absorbance of CI and DI.

*Isorotundifoline.* Differentially, as individual alkaloid.

1 AII, using CII as reference solution.

2 BIII, using DII as reference solution.

3 CI, using AI as reference solution.

4 DI, using AI as reference solution.

5 By subtracting the absorbance of AI from CI or DI.

In association with mitraphylline

6 AII, using blank silica gel eluate as reference solution.

7 BIII, using AIII as reference solution.

*Mitraphylline.* As individual alkaloid.

1 CII, using blank silica gel eluate as reference solution.

2 DII, using CIII as reference solution.

*Isomitraphylline.* As individual alkaloid.

1 BII, using silica gel eluate as reference solution.

2 AI, using BI as reference solution.

3 By subtracting the absorbance of BI from the absorbance of AI both read against blank silica gel eluate as reference solution.

*Rhynchophylline.* As individual alkaloid.

1 AIII, using silica gel eluate as reference solution.

2 CIII, using silica gel eluate as reference solution.

3 BIII, using AII as reference solution.

4 DII, using CII as reference solution.

5 By subtracting the absorbance of AII from the absorbance of BIII.

*Isorhynchophylline.* As for rotundifoline.

Table XIV gives the results of the analyses of a six component mixture by the three spectrophotometric methods discussed, a comparative summary being given in Table XV.

ACKNOWLEDGEMENT

One of us (MZA) thanks the British Council for a Colombo Plan Scholarship to enable him to undertake research work at the Chelsea College of Science and Technology.

SUMMARY

Ultraviolet spectrophotometry has been employed for the quantitative determination of six Mitragyna oxindole alkaloids after separation by TLC and subsequent elution from the layers. An investigation has been undertaken of all the factors affecting complete recovery of the alkaloids and elimination of interfering substances. The method known as differential spectrophotometry has been applied to mixtures of alkaloids which are difficult to separate completely by TLC.

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