THE ESTIMATION OF MORPHINE, CODEINE AND THEBAINE IN OPIUM AND IN POPPY LATEX BY PAPER CHROMATOGRAPHY

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The recommended method involves separation of the morphine from the mixed alkaloids in chloroform using 0·1N sodium hydroxide and separation of the codeine and thebaine by means of a buffer solution of pH 2·0. The two fractions are then chromatographed on paper and after suitable colour development the areas of the spots are measured planimetrically. Constant areas for the same quantities of alkaloid are obtained by ensuring that the initial spots of material applied to the starting line are of constant area and that the running conditions are rigidly controlled, especially the temperature. To overcome the variability due to differences between sheets of chromatographic paper the "4 point bioassay" technique is used (in which two dose levels of standard solution of alkaloids and two dose levels of the unknown fraction are applied for each assay). The coefficients of variation for individual assays are for morphine, 3 to 6 per cent; for thebaine 5 to 7 per cent. Replicate assays can readily be run on one sheet of paper resulting in increased accuracy. The method is particularly suitable for small quantities of raw material (100 to 200 mg.) and is two to three times quicker than other published methods.

DURING an investigation of the possible function of the alkaloids of *Papaver somniferum* L., a rapid assay suitable for the main alkaloids in small samples of fresh latex was required. Published methods (Pfeifer, 1958; Milos, 1960) were time consuming, and of poor reproducibility due to the difficulty in removing contaminants (cf. Pride and Stern, 1954). Attempts to isolate the alkaloids by paper chromatography were tedious and frequently required larger amounts of raw material than were available.

An adaptation of the method of Fairbairn and Suwal (1959) for *Conium* has been found to give consistent results and we believe it to be of more general applicability than the original method.

EXPERIMENTAL

Preliminary work showed that attention to the following points was necessary to ensure consistent results.

(i) *Running solvent.* A single phase system with fixed proportions of individual solvents, as recommended by Smith (1960) and Betts (1961), was used.

(ii) The paper must be washed with running solvent.

(iii) *Running conditions.* Rigid temperature control is essential to prevent elongation and overlapping of the spots. Optimum separation and shape of the spots are obtained at 18° (±1°) and when the solvent front moves about 25 cm. in 18 hr.

Application of the alkaloids. We confirmed previous experience (Fairbairn and Suwal, 1959) that the spots of material applied to the starting line must be of constant area. Details of how this is achieved are incorporated in the method.

Area quantity curve. Over a range of quantities which produced areas between about 3 and 7 cm.^{2} the area was proportional to the log quantity of alkaloid applied. Fig. 1 shows two area/log quantity curves for morphine representing the highest and lowest of nine curves, each based on separate sheets of paper. This satisfactory area/log quantity relationship allowed us to use the "4 point bioassay" method (Fisher, Parsons and Morrison, 1948; Ohtsu and Mizuno, 1952; Fairbairn and Suwal, 1959).

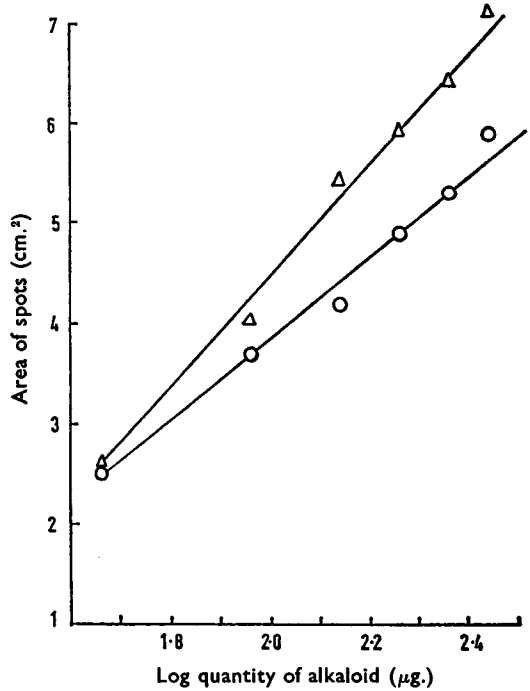

FIG. 1. Area/log quantity curves for morphine; each curve represents the results obtained on one sheet of paper. The two curves are the highest and lowest of nine curves, each from a separate sheet of paper, and thus indicate the range of the results for nine separate sheets of paper.

Recommended Method

Preliminary fractionation of the alkaloids from raw material is necessary for two reasons. Firstly the thebaine spot overlaps those of papaverine and the minor alkaloids. Secondly, the proportion of morphine is so much higher than that of the other alkaloids that the necessary quantity of extract cannot be applied to produce suitable spot areas for all the alkaloids simultaneously. Separation of the morphine is based on its solubility in 0·1N NaOH; traces of other phenolic alkaloids, such as

narceine and narcotoline, are removed in the later solvent extraction and paper chomatographic stages. Codeine and thebaine are separated from a chloroform solution of the remaining alkaloids by shaking with a buffer solution of pH 2·0; using artificial mixtures this gave 99 per cent recovery in contrast to only 75 per cent recovery when 0·2 per cent tartaric acid was used, as recommended by Pfeifer (1958).

Accurately weigh about 100 mg. of powdered opium and triturate with a few ml. of acid methanol (methanol 70 ml., hydrochloric acid B.P. 1 ml., water to 100 ml.). Filter and transfer to a 20 ml. volumetric flask; wash the filter with further solvent and make up to volume. For fresh latex, use 100 to 200 mg., add about 8 ml. of the acid methanol, stir, filter and make up to volume as above in a 10 ml. volumetric flask. Pipette 4·0 ml. of the acid methanol extract into a separating funnel containing 10 ml. 0·1N sodium hydroxide and shake for 1 min. Extract with 25, 15, and 10 ml. portions of chloroform, shaking for about 3 min. on each occasion, wash the combined chloroform layers with two 5 ml. portions of 0·1N sodium hydroxide and add these to the aqueous layer.

(i) To the *aqueous layer* add 0·5 ml. hydrochloric acid B.P. followed by 2 ml. solution of ammonia 10 per cent. Extract with 25, 20 and 15 ml. portions of ethyl acetate and wash the combined ethyl acetate layers with two 5 ml. portions of water. Reject the washings and evaporate the ethyl acetate extract to dryness on a water-bath. Dissolve the residue in methanol and make up to a volume of 1 ml. Use this solution, which contains the morphine, for the chromatographic procedure described below.

(ii) Extract the *chloroform layer*, with three 15 ml. portions of a buffer solution of pH 2·0 (M/5 KCl, 50 ml.; N/5 HCl, 10·6 ml., water to 200 ml.) shaking for about 5 min. on each occasion. Reject the chloroform layer, combine the acid layers and add 5 ml. N sodium hydroxide. Extract with 30, 25 and 15 ml. portions of chloroform and wash the combined chloroform layers with two 5 ml. portions of water. Reject the washings and evaporate the chloroforom extract to dryness on a water-bath. Dissolve the residue in methanol and make up to a volume of 1 ml. This solution contains the codeine and thebaine.

Standard solutions (*a*) morphine base: 1 per cent in methanol; (*b*) codeine base: 0·3 per cent; thebaine base: 0·2 per cent, in methanol.*

Use 0·01 ml. and 0·02 ml. of these solutions as standards.

Application of the solution to the paper. Sheets of Whatman No. 1 paper (46 × 54 cm.) washed with running solvent by descending technique for about 18 hr. and then dried at 100° are used with the direction of the fibres vertical.

With a cork borer of 7 mm. diameter make light impressions on the starting line, 3–4 cm. apart, thus ensuring uniformity of the spots. For each assay use 4 of these areas as follows; Standard S_1 (0·01 ml.); Unknown U_1 (0·01–0·05 ml. for morphine, double this volume for codeine and thebaine, so that the quantities in U_1 approximate those in S_1); Standard S_2 (0·02 ml.); Unknown U_2 (0·02–0·1 ml.). Apply the solutions

* As codeine and thebaine are estimated simultaneously, a *mixture* of the two alkaloids is used as a standard (Fairbairn and Suwal, 1959).

with an Agla syringe under constant drying conditions (Fairbairn and Suwal, 1959), adding sufficiently small quantities at a time to avoid exceeding the marked areas.

Development and Measurement of the Spot Areas

Running solvent. n-Butanol: acetic acid: water (5:1:2). Run the chromatogram in a tank maintained in a suitable water-bath at a temperature of 18° (±1°) for 18 hr. (±0·5 hr.). Dry the paper at 80° (10 min.) and spray with Dragendorff's reagent (Munier and Macheboeuf, 1951). Outline the spot areas in pencil while still wet and dry the papers in a current of warm air. Measure the outlined areas with a planimeter and calculate the amount of alkaloid from the following equation.

$$\log r = \frac{(U_2 + U_1) - (S_2 + S_1)}{(U_2 - U_1) + (S_2 - S_1)} \times \log 2.$$

Where r = ratio of quantities of alkaloid in Standard and Unknown spots.
S_1 and S_2 = spot areas corresponding to amounts of alkaloid in solutions of Standard applied to the paper.
U_1 and U_2 = spot areas corresponding to amounts of alkaloid in solutions of Unknown applied to the paper.

Results

An artificial extract was prepared by dissolving known quantities of morphine, codeine, thebaine and papaverine in acid methanol. Papaverine was included to represent the alkaloids whose R_F values overlapped those of codeine and thebaine. Four or five separate assays, each involving three sets of area measurements on the same sheet of paper, were made by the recommended method. The results in Table I are typical of the variation of spot area within and between papers.

TABLE I

REPLICATE ASSAYS FOR MORPHINE IN ARTIFICIAL EXTRACT

(Actual amount present = 40·5 mg./20 ml.)

Chromatographic paper	Area of spots in cm.²				Quantity of morphine (mg./20 ml.)
	S_1	S_2	U_1	U_2	
1	3·5	5·2	3·8	5·0	38·7
	3·6	5·0	3·7	5·0	38·8
	3·9	5·3	4·1	5·3	40·0
2	3·2	5·0	3·2	5·0	37·8
	3·4	5·4	3·5	5·4	38·5
	3·5	5·4	3·7	5·4	39·3

The results for morphine are: present, 40·5 mg., found (mean of 15 assays) 39·4 mg. s.d. 1·12; for codeine: present, 18·45 mg., found (mean of 15 assays) 18·24 mg. s.d. 0·55; thebaine: present, 8·93 mg., found (mean of 12 assays) 8·83 mg. s.d. 0·44.

Powdered opium. Three separate assays, each involving four sets of area measurements, were made on a sample of powdered opium. Recovery assays were also made by adding known quantities of alkaloid to the powdered opium and re-assaying. The results are shown in Table II.

TABLE II

SUMMARY OF RESULTS OF REPLICATE ASSAYS OF OPIUM AND OF OPIUM TO WHICH KNOWN QUANTITIES OF ALKALOID WERE ADDED. RESULTS EXPRESSED AS PER CENT ALKALOID

	Morphine	Codeine	Thebaine
Opium			
Mean of 13 assays	8·79	1·25	1·07
Standard deviation	0·53	0·076	0·073
Coefficient of variation	6·0 per cent	6·1 per cent	6·8 per cent
Opium + alkaloid 1			
Original + added amount	9·86	1·51	1·32
Amount found (mean of 4 assays)	10·37	1·54	1·31
Opium + alkaloid 2			
Original + added amount	19·50	2·57	2·30
Amount found (mean of 4 assays)	19·61	2·36	2·18

DISCUSSION

Reproducibility and Accuracy

The reproducibility of the method depends on producing constant areas of the running spot for identical quantities of the same alkaloid. To achieve this we have attempted to control every variable involved, but the differences between sheets of paper could not be eliminated. Table I shows the variation in spot area which is typical for a given quantity of morphine; for five sheets of paper it is 6 to 8 per cent of the mean. Fig. 1 represents the extreme variation for nine sheets of paper (8 to 10 per cent of the mean). This contrasts with our earlier experience with coniine (Fairbairn and Suwal, 1959) where a variation of up to 35 per cent of the mean using 4 sheets of paper occurred. This improvement may largely be due to the rigid temperature control. The results show that there still remains significant variation between different sheets of paper however, and by using the "4 point bioassay" technique a considerable increase in reproducibility may be obtained. For an artificial extract the standard error of the mean of four assays ($P = 0{\cdot}95$) is for morphine and codeine ± 3 per cent; for thebaine ± 5 per cent. For powdered opium the corresponding figures for morphine and codeine are ± 6 per cent and for thebaine ± 7 per cent. The *accuracy* of the method is shown by the recovery experiments in Table II, and the 97–99 per cent recovery obtained with the artificial extract.

Since few previous workers quote adequate limits of error it is difficult to compare our method with theirs. However, Römisch (1961) who used a method involving elution of chromatographic spots followed by a spectrophotometric assay, quotes percentage errors for artificial extracts and for opium. Means of 3 assays of the artificial extract gave for morphine, errors of -5 to -8 per cent; for codeine 0 to -10 per cent and for thebaine -16 per cent. For opium (means of 2 assays) the errors were slightly greater ranging up to -17 per cent for thebaine.

Convenience

A certain amount of judgment is required in outlining the spots after spraying but results obtained by workers with no previous experience of the method show that the ability to do this consistently is rapidly acquired. The planimetric measurement of the final spot areas is the main time-consuming item; however we have found that, ignoring the overnight run in the chromatographic tank, the method is two to three times quicker than the other methods referred to earlier.

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References

Betts, T. J. (1961). Thesis Univ. of London, p. 57.
Brockmann-Hanssen, E. (1955). *Medd. norsk. farm. Selk.*, **17**, 76–86.
Fairbairn, J. W. and Suwal, P. N. (1959). *Pharm. Acta Helvet.*, **34**, 561–7.
Fisher, R. B., Parsons, D. S. and Holmes, R. (1949). *Nature Lond.*, **164**, 183.
Milos, C. (1961). *J. pharm. Sci.*, **50**, 837–39.
Munier, R., and Macheboeuf, M. (1951). *Bull. Soc. Chim. biol.*, **33**, 846–856.
Ohtsu, T. and Mizuno, D. (1952). *Jap. J. med. Sci. Biol.*, **5**, 37–44.
Pfeifer, S. (1958). *Bull. Narcotics*, **10**, 18–33.
Pride, R. R. A. and Stern, E. S. (1954). *J. Pharm. Pharmacol.*, **6**, 590–606.
Römisch, H. (1961). *Pharmazie*, **16**, 373–7.
Smith, I. (1960). *Chromatographic and Electrophoretic Techniques.* Vol. I, p. 20. London: Medical Books Ltd.

The paper was presented by Miss Wassel.