ALKALOIDS OF *RAUWOLFIA* SPECIES

PART II.* THE ESTIMATION OF RESERPINE IN SAMPLES OF RAUWOLFIA BY MEANS OF COUNTERCURRENT DISTRIBUTION

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SEVERAL instances of the use of countercurrent distribution for the separation of alkaloids in various species of *Rauwolfia* have been reported[1–5], and the method has been useful both in the initial resolution of extracts into groups of alkaloids[3], and in the purification of individual substances intractable to other approaches[4]. While exploring the possibilities of this technique, the distribution characteristics of reserpine have been investigated in a variety of systems and applied to the estimation of this alkaloid in small samples of several species of *Rauwolfia*.

A number of methods have been described for the assay of reserpine, but some of these are designed for use with pharmaceutical preparations in which reserpine preponderates, and are less suitable when dealing with natural alkaloidal complexes. Sakal and Merrill[6] have, however, assayed reserpine in crude extracts by ultra-violet spectrophotometry after an initial separation by paper ionophoresis in 5N acetic acid, while Dechene[7] has described a fluorimetric method which was applied to an extract of *R. serpentina*. In the latter case it was not established that the extraction procedure separated reserpine completely from other fluorescent alkaloids, while it has been our experience that reserpine and rescinnamine are not adequately separated by paper electrophoresis in 3N acetic acid. High capacity paper chromatography, with 2 per cent acetic acid in propylene glycol as stationary phase and 1 : 1 benzene *cyclo*hexane as mobile phase enabling as much as 5 mg. of reserpine to be run without bad streaking, has also been used for a preliminary separation of reserpine from crude extracts before assay by ultra-violet absorption[8], while a more recently published method[9] makes use of liquid-liquid partition chromatography on a Celite column followed by hydrolysis and estimation of the resulting trimethoxybenzoic and trimethoxycinnamic acids.

A study was made of the distribution of reserpine in several two phase systems consisting of (*a*) various organic solvents with aqueous acetic acid, and (*b*) an ether-chloroform (3 : 1) mixture with aqueous buffer solutions. Using the latter system, the log of the partition coefficient varied in a linear manner with pH over the range pH 2·4–3·2, giving a partition coefficient of 1 at pH 3·1. At this pH, the partition isotherm was linear over the concentration range 0·1–0·4 per cent w/v reserpine in the solvent phase. A 24 transfer distribution of a sample of reserpine in a Gilson-Wright semi-automatic countercurrent apparatus yielded a distribution curve (Fig. 1) symmetrical about the centre point, the amount

* Part I, *Chem. and Ind.*, 1955, 1481.

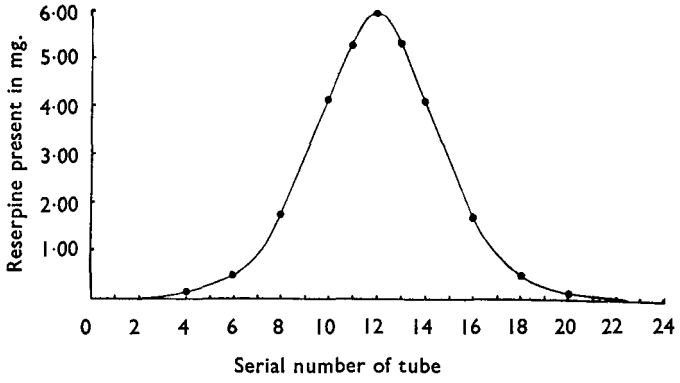

FIG. 1. A 24-transfer countercurrent distribution of pure reserpine.

of solute in each tube being determined by ultra-violet absorption spectroscopy. Under similar conditions, a 14 transfer distribution of a reserpine-rich fraction from *R. vomitoria* in a smaller apparatus yielded a distribution curve in which the central reserpine peak was over-lapped on either side by other absorbent material (Fig. 2). By making three additional transfers and discarding three issuing fractions, the resolution was improved sufficiently to obtain a distinct reserpine peak (Fig. 3), which could then be used to estimate the amount of this alkaloid present. The essentially homogeneous nature of the material comprised within this peak was checked by paper chromatography and electrophoresis. The technique was applied to several different rauwolfia samples with the results given below. It was found desirable to use a chloroform-soluble fraction, and this was conveniently prepared from the root powder by a method similar to that described by Hochstein, Murai and Boegemann[3] for *R. heterophylla.*

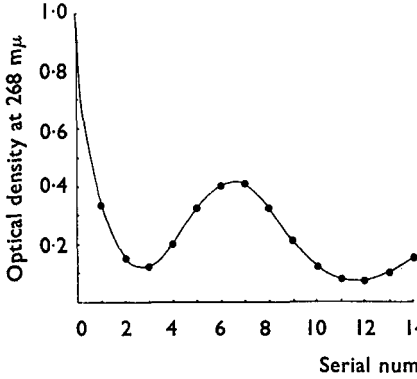

FIG. 2. A 14-transfer countercurrent distribution of a fraction from *R. vomitoria* showing incomplete resolution of reserpine.

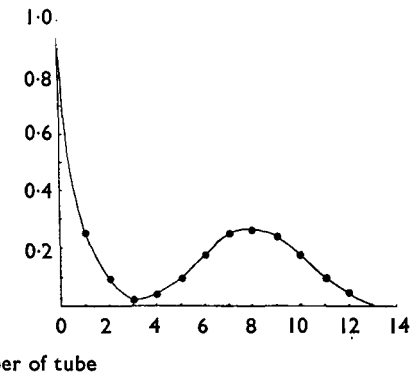

FIG. 3. Effect of withdrawing 3 further fractions from 15-tube apparatus giving adequate resolution for estimating reserpine in *R. vomitoria.*

EXPERIMENTAL

Countercurrent Distribution of Reserpine

The solvent system used was as follows.

The mobile phase was composed of chloroform (250 ml.) diluted to 1 litre with ether.

The stationary phase consisted of buffer solution of pH 3·1 containing 16·3 g. of citric acid ($C_6H_8O_7 \cdot 1H_2O$) and 16·1 g. of disodium hydrogen phosphate ($Na_2HPO_4 \cdot 12H_2O$) in 1 litre of water.

These solutions were mutually saturated before use and the stationary phase put in 25 tubes of a semi-automatic Gilson-Wright apparatus with a lower phase capacity of 20 ml. per tube. After carrying out one transfer with 20 ml. of mobile phase to ensure equilibrium, 20 ml. of a solution of reserpine (100 mg.) in chloroform (12·5 ml.) diluted to 50 ml. with ether was introduced and distributed through the apparatus using about 50 tube-inversions and a settling time of 2 minutes per transfer. After 24 transfers, 1 ml. of ammonia (sp.gr. 0·880) was added to each tube, the apparatus given 50 tube-inversions and aliquots of 5 ml. withdrawn from each upper phase and diluted to 50 ml. with pure ethanol. The optical density of each sample was measured at 268 mμ using as blank solvent a portion of equilibrated mobile phase (5 ml.) similarly diluted with ethanol.

This experiment was repeated with a 15 tube manually operated apparatus of similar design using 30 tube-inversions per transfer. Using the previously determined value of log ϵ 4.21 for the reserpine maximum at 268 mμ, the estimated quantity of reserpine in the apparatus was 38·7 mg. (97 per cent of the amount taken).

Preparation of a Reserpine-rich Fraction from Rauwolfia Root

The following example, using a commercial sample of *R. vomitoria*, illustrates the general procedure adopted in all estimations.

A suspension of the milled root-powder (50 g.) in methanol (500 ml.) was gently refluxed on the steam bath for 2 hours. After filtration, the extraction was repeated three times giving eventually a nearly colourless solution. The combined extracts were concentrated at reduced pressure to about 15 ml. and the residual solution added to N acetic acid (30 ml.) to give a turbid solution from which fatty material was removed by two washings with *n*-hexane (25 ml.). The clear liquid was treated at 5–10° with ammonia to pH 8–9, the tan-coloured precipitate collected, washed with water and combined with an additional quantity extracted by chloroform from the filtrate. The total weak bases (1·4 g.) were now extracted alternately with chloroform and N acetic acid, the extracts shaken together and the chloroform layer separated, washed with a little ammonia and evaporated, affording a brown powder (0·69 g.).

Countercurrent Analysis of Reserpine-rich Fractions

To estimate the reserpine content, about 100–130 mg. of the brown powder was accurately weighed into chloroform (6·25 ml.) and the solution diluted with ether to 25 ml. After centrifuging to remove a light

flocculent precipitate, a part (20 ml.) of the clear supernatant liquid was pipetted into the 15-tube countercurrent apparatus. The procedure described for pure reserpine was now followed except that 17 transfers were given, withdrawing three fractions from the outlet. After adding ammonia to each tube and shaking, aliquots of the upper phases were taken and optical densities measured at 268 mμ. The reserpine content was calculated from the densities of the tubes contained within the peak (see Fig. 3), and the combined upper layers of these tubes then evaporated to dryness. The residue was compared with pure reserpine (*a*) by ascending paper chromatography on Whatman No. 20 paper using 10 per cent acetic acid in 5 per cent aqueous sodium acetate just saturated with di(*n*-butyl)ether[10], and (*b*) by paper electrophoresis on Whatman No. 31 Extra Thick paper in 3N acetic acid at 400 volts at 10 milliamps for about 4 hours. The papers were examined for fluorescence under ultra-violet light.

Results and Discussion

The method described above was applied to a commercial sample of *Rauwolfia serpentina* and to two distinct samples of *R. vomitoria.* Since these materials were also extracted on a larger scale in 5 kg. batches, it is possible to compare the estimated reserpine contents with the quantities actually isolated and these figures are given in Table I. The isolation of reserpine by several alternative routes was examined, and the yields quoted are those obtained by the most quantitative procedure encountered, viz. extraction with 10 per cent acetic acid of a water-washed methanolic root extract, followed by transference of reserpine from the acetic acid into chloroform and finally chromatography on alumina[11].

TABLE I

Comparison of estimation results with yields of reserpine by large scale extraction

Material	Weight of reserpine in 5 kg. of sample	
	Estimated by countercurrent	Actually isolated
R. serpentina	2·7 g.	2·6 g.
R. vomitoria (Sample I) ..	7·0 g.	7·0 g.
R. vomitoria (Sample II) ..	12·2 g.	10·9 g.

The method is thus capable of indicating the approximate yield of reserpine which may be expected from rauwolfia samples of the species mentioned, and is conveniently operated, since a sufficient degree of resolution is obtainable with comparatively few transfers. The combined use of paper electrophoresis and paper chromatography under the conditions described has been found to discriminate between a large number of rauwolfia alkaloids, including for example, reserpine and rescinnamine, and thus provides a fairly rigorous indication of the homogeneity of the material used for assay. In practice, reserpine provided the sole strongly fluorescing spot, and the one other barely discernible spot which could be

detected in two cases was of such a low order of intensity that it is not likely to have interfered with the estimation of reserpine within the limits of accuracy required. In the case of other species of *Rauwolfia* in which the reserpine content may be of an altogether lower amount, the method as described can give some indication of this amount, but the contributions of any overlapping alkaloids are likely to be proportionately greater. An experiment carried out with a Brazilian sample of *R. sellowii*, for example, gave a much reduced reserpine peak from which it was possible to calculate that the reserpine content was less than 0·004 per cent. A more accurate figure could not be derived since paper electrophoresis revealed that two other substances were present within the peak. Hochstein has reported[12] a yield of 0·002 per cent of reserpine in this species.

In cases of inadequate resolution in 17 transfers, the accuracy of the procedure could presumably be increased by carrying out more transfers but this has not been found necessary within the scope of the work reported.

Summary

1. The behaviour of reserpine in a countercurrent apparatus when distributed between a number of systems and especially ether-chloroform (3 : 1)/buffer of pH 3·1 has been examined.

2. This is the basis of a convenient method for the estimation of reserpine in small samples of *Rauwolfia serpentina* and *Rauwolfia vomitoria*, and the results have been compared with the yields of reserpine isolated from larger quantities of the same material.

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