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The determination of thebaine in *Papaver bracteatum* Lindl by gas-liquid chromatography

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Several solvents have been examined in the process of developing a convenient method for extracting powdered material and removing interfering pigments to produce an extract of *Papaver bracteatum* suitable for g.l.c. assay. The most suitable extracting solvent was aqueous acetic acid (5%); the assay method gave satisfactory reproducibility with a wide range of plant materials (coefficient of variation 0·63 to 3·33%). The accuracy was checked by recovery experiments with pure thebaine and by examination of exhausted marcs. The presence of other alkaloids did not interfere with the assay. There were substantial differences in the amounts of thebaine extracted from capsule and root samples by ammoniacal methanol and by aqueous acetic acid, indicating the presence of thebaine in a "bound" form. The minimum amount of thebaine that could readily be determined by the g.l.c. method was 0·3 mg in the sample of powder used for the assay.

Papaver bracteatum may be an important source of opiates because of its high thebaine content (Fairbairn & Hakim, 1973; United Nations, 1973a, b). An efficient and convenient method of determining thebaine, not only in the root, but in the more highly pigmented capsule and leaf, is urgently required. The colorimetric method of Sakuri (1960) was used by Böhm (1965), but we found it tedious as each of the several stages involved exact timing. Fairbairn & Hakim (1973) used quantitative t.l.c. followed by spectrophotometry, but occasionally unusual results were obtained due to contamination from the adsorbent. Highly pigmented material also required extensive pretreatment to remove interfering pigments.

A simple method of removing pigment and extracting the alkaloids was devised using ammoniacal methanol (Fairbairn, 1973) and consistent results were obtained; however, Prof. C. A. Salemink (personal communication) found that higher yields of alkaloid could be obtained if an acid solvent was used. Other workers reported similar results (Cheng, 1972; Vincent & Gentner, 1974) and several different solvents and procedures have been suggested. We decided to examine these in detail on a wide variety of plant material and to devise a method of assay which is convenient and gives reproducible results.

MATERIALS AND METHODS

Materials

Capsule samples UNB-6 and UNB-17 were obtained through the United Nations, Geneva. Root and leaf samples UNB-4 (seeds obtained from United Nations, Geneva), latex samples MH-3 (seeds obtained from the Royal Botanic Gardens, Kew)

and capsule samples for duplicate assays were collected from plants grown at our experimental gardens in London.

Initial extracting solvent

Samples of plant material were extracted with different solvents and the extracts treated as described in the *Recommended Method*, the thebaine content being determined by g.l.c. (see below).

Methanol–0·880 ammonia (98:2), used as recommended by Fairbairn (1973) gave consistent results with samples of capsule, root and leaf (Table 1) giving coefficients of variation of 3·0 to 4·2%. Pure thebaine, when passed through the process gave 99% recovery, thebaine added to a known sample gave 101% recovery. Furthermore, the marcs left after assaying three samples of UNB-6 were combined and re-extracted with the solvent. The resulting peak on the g.l.c. record was too small for accurate measurement but at most would represent a thebaine content of only 0·005% in the marc, against a value of 1·24% before extraction (Table 1).

Table 1. *Thebaine content of various plant samples extracted with either MeOH–0 88 NH_4OH (98:2) or 5% aqueous acetic acid.* All extractions at room temperature unless otherwise stated.

Sample	Solvent	No. of assays	Mean % thebaine content
Capsule, UNB-6	MeOH–NH_4OH	10	1·24
	Acetic acid	10	1·36
Capsule, UNB-17	MeOH–NH_4OH	6	2·80
	Acetic acid	2	3·30
	Acetic acid; 60°	3	3·27
Root, UNB-4	MeOH–NH_4OH	5	0·59
	Acetic acid	5	0·77
Leaf, UNB-4	MeOH–NH_4OH	5	0·102
	Acetic acid	5	0·099
Dried latex, MH-3(1)	MeOH–NH_4OH	2	34·54
	Acetic acid	2	35·66
Dried latex, MH-3(2)	MeOH–NH_4OH	2	34·55
	Acetic acid	2	34·16

Aqueous acetic acid 5% at room temperature (20°) was used on the same plant samples, and gave higher yields of thebaine with capsule and root materials; extraction with warm acetic acid (60°) gave no increase in the quantity of thebaine extracted (Table 1). Experiments using thebaine added to capsule material gave 98 to 101% recovery; re-extraction of the marcs after assay, with either 5% aqueous acetic acid or methanol–ammonia, (98:2) yielded no further thebaine.

Samples of latex gave no significant difference in thebaine content when extracted by either of the above two methods; however, the capsule pericarps, from which the latex had been obtained, still contained significant amounts of thebaine. Extraction of nine samples of these bled capsules, from four varieties of *P. bracteatum*, showed that the acid extraction method gave 49, 170, 100, 79, 100, 107, 100, 73 and 65% more thebaine respectively than using the MeOH–NH_4OH process. With some samples the marc left after exhausting with MeOH–NH_4OH was re-extracted with acetic acid and the additional alkaloid obtained confirmed to be thebaine by comparison with pure thebaine, using g.l.c., t.l.c. and mass spectrometry of the alkaloid eluted from the thin-

layer plates. In contrast, the expelled latex yielded no further thebaine to acid after previous extraction with MeOH–NH_4OH.

Other solvents. Sulphuric acid (2%) used on four samples of capsule gave significantly lower values than the acetic acid method; citric acid (3%) gave similar but slightly lower results than acetic acid. No advantage was gained by using more concentrated acetic acid solutions for extraction.

We therefore recommend the use of *5% aqueous acetic acid* for the initial extraction. Using the procedure described below, even deeply pigmented leaf material can be satisfactorily assayed. We have also confirmed the suggestion (Cheng & Doorenbos, 1973) that drying at 105° rather than 60° (Fairbairn, 1973) leads to no loss of thebaine and is more convenient.

Recommended method

Freshly collected material should be air-dried at 105° and powdered so that all the material passes through a mesh of not greater than 0·5 mm aperture (500 μm mesh). The amount of powder and the volume of the final extract should be varied according to the expected potency.

Accurately weigh 0·1 to 1·0 g of powder and shake for 30 min with 15 ml of 5% aqueous acetic acid. Allow to settle, pipette off the supernatant and filter, retaining the filtrate. Add a further 2 × 10 ml of solvent to the residue and shake each for 15 min; pipette and filter as before. Wash the residue and filter with a further 5 ml of the solvent. Add excess NH_4OH to the combined filtrates and extract with 4 × 10 ml $CHCl_3$ gently evaporating each extract to dryness in the same glass basin on a water bath. Dissolve the residue in 2·0 to 5·0 ml of the following solution: cholesterol acetate 80 mg in 100 ml of ethanol. Use 4 to 5 μl for g.l.c.

For g.l.c. we used a Pye 104 instrument with flame ionization detector: column, length 5 ft, 4 mm i.d. was packed with 2% OV 17 on Chromosorb W (AW-DMCS, 80–100 mesh) and operated at 270° with a nitrogen flow rate of 60 ml min^{-1}. Peak area was estimated by measurement of height × (width at half height).

Statistics. Replicate assays were made on samples of capsule, root and leaf. A further 17 samples of capsule and 3 samples of dried latex were analysed in duplicate and the coefficient of variation calculated by a method previously described (Fairbairn & Liebmann, 1973). The coefficients of variation for all samples ranged from 0·63 to 3·33% (Table 2).

Table 2. *Results for replicate assays of various plant samples using the Recommended Method.*

Sample	No. of assays	Mean % thebaine content	Coefficient of variation
Capsule, UNB-6	10	1·36	2·32%
Capsule, UNB-17	5[a]	3·28	1·43%
Capsule (Bled pericarp)	17 × (2)	0·20 to 2·28	2·12%
Dried latex	3 × (2)	30 to 36	0·63%
Root, UNB-4	5	0·77	2·16%
Leaf, UNB-4	5	0·099	3·33%

[a] Includes results at 60° (see Table 1).

DISCUSSION

The recommended method has given satisfactory reproducibility with capsule, root, leaf and latex, and a wide range of thebaine contents (0·1% to 36%). The coefficients of variation for single assays ranged from 0·63 to 3·3%. The accuracy was almost 100% by recovery experiments and by examination of the marcs left after the assay process. No decomposition of the thebaine seemed to take place since almost 100% recovery was obtained using added thebaine or thebaine alone. The operating time for the assay is about 2 h, including the dead time involved in the shaking and the g.l.c. stages.

Is there a "bound" form of thebaine present?

After completely exhausting certain samples with ammoniacal methanol, it is possible to obtain more thebaine by further treatment with dilute acetic acid. The consistent results from the alkaline treatment indicate that a definite amount of a normal thebaine salt is present; the additional thebaine obtained by further acid treatment must be bound in an unusual manner. Apparently the two forms are not always present as we found no difference between alkaline and acid treatment with leaf or expelled latex. Similarly Cheng's results (1972) showed little difference with stem, bract or root, but distinct differences in the capsule, as we also found. Our examination of the capsule showed that the expelled latex had only the one form of thebaine, but the bled capsule had significant amounts of the "bound" form. In this tissue the acid extract yielded 49 to 170% more thebaine than the alkaline extracts. Some preliminary investigations indicate that the binding of the thebaine may be more complex than originally anticipated; water, at a neutral to alkaline pH, liberates thebaine from an ammoniacal methanol exhausted marc, but to a lesser extent than that liberated by aqueous acetic acid.

In our experience acetic and citric acids were effective but sulphuric acid gave unsatisfactory results. In the conditions we used, the products formed after sulphuric acid extraction, were not sufficiently soluble in the final ethanolic internal standard solution for accurate results; in the very different conditions recommended by Vincent & Gentner (1974) this disadvantage of sulphuric acid may not arise.

Presence of other alkaloids

Isothebaine is claimed to be present in certain varieties of this plant and in the closely related species *P. orientale* (Böhm, 1967; Santavy, 1970), alpinigenine (Guggisberg, Hesse & others, 1967) and oripavine (Kuhn & Pfeifer, 1963) may also be present. In our g.l.c. system these alkaloids have the following relative retention times (cholesterol acetate 1·00 = 6·6 min); oripavine–0·36; thebaine–0·42; isothebaine–0·64 and alpinigenine–0·89. Despite the proximity of thebaine and cholesterol acetate to oripavine and alpinigenine respectively, in over 250 assays so far performed, no interference has been found between them. In line with previous experience none of the commonly occurring opiates of the morphinane and benzylisoquinoline series were detected.

The *Recommended Method* therefore has a satisfactory reproducibility and accuracy, and separates the thebaine from other alkaloids likely to be present.

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