Colorimetric Determination of Thebaine in *Papaver bracteatum*

KOSTANTIN IKONOMOVSKI

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Abstract □ A rapid colorimetric test for the determination of thebaine in dried, extracted *Papaver bracteatum* is described. The method is based on the development of an orange color formed by the nitroso color reaction for phenols. The absorption peak is near 450 nm. The linear range response is from 0 to 1.0 mg of thebaine. The reproducibility of the method is quite good, making it useful for process evaluation and in a selection program for the cultivation of *P. bracteatum* for optimum thebaine content.

Keyphrases □ Thebaine—colorimetric determination in *Papaver bracteatum* □ *Papaver bracteatum*—colorimetric determination of thebaine in extract □ Alkaloids—thebaine, colorimetric determination in extract from *Papaver bracteatum*

A simple and rapid analytical method using small samples was needed to follow the process of thebaine isolation from *Papaver bracteatum* as well as for a selection program for the cultivation of this new alkaloidal raw material. GLC assays (1–9) have received the most attention, and high-pressure liquid chromatographic methods were reported recently (10, 11). Since not every laboratory is equipped with these rather expensive instruments, the colorimetric method described here was developed.

BACKGROUND

When thebaine (I) is heated with mineral acids, the ether linkage in the molecule is cleaved, resulting in a phenolic hydroxyl group in position 4. Color reactions for phenols then may be used for the determination of the formed phenolic alkaloids.

Some attractive, rapid colorimetric methods (12–14) are based on conversion of thebaine to phenolic alkaloids followed by the color reaction immediately after extraction of the plant material, but they yield variable results. Pfeifer's colorimetric method (15) for the determination of morphine in *Papaver somniferum* is widely used for process control in the isolation of morphine. To develop a similar method for the determination of thebaine in *P. bracteatum*, preference was given to the nitroso color reaction for phenols (16) over the more lengthy Sakurai method (17), which relies on the formation of an ammoniacal potassium ferricyanide color complex.

Purification of the plant extracts is unavoidable. TLC separation (18, 19) followed by removal of the thebaine spot, prior to its degradation and color reaction, is time consuming. Organic solvent extraction of thebaine, often used prior to GLC assays (1, 2, 4, 5, 9), from an alkaline aqueous extract of *P. bracteatum* yields solutions pure enough for the colorimetric procedure described in this report.

EXPERIMENTAL

Plant Culture—The seeds were obtained from the United Nations Narcotics Laboratory[1].

Reagents—All chemicals were reagent grade. The aluminum oxide was acidic[2] chromatographic grade. The thebaine standard was prepared in the laboratory with a melting point of 194° and a single TLC spot.

Sample Preparation—*P. bracteatum* poppy plants were chopped or broken into small pieces and dried at 105°. Dried samples were milled and screened to <1 mm (~20 mesh).

Procedure—Approximately 100 mg of sample was weighed accurately, mixed with 0.5 ml of water, and allowed to stand for 15 min. Acidic aluminum oxide (2 g) was added and mixed to form a dry powder. The mixture was added to a glass column (~150 × 13 mm i.d.) containing another 2 g of aluminum oxide held in place with a cotton plug. The thebaine was eluted from the column with 30 ml of 5% (v/v) acetic acid at a flow rate of ~1 ml/min into a separator. The pH of the eluate was adjusted to >11 with 10% (w/v) NaOH (~40 ml).

The aqueous phase was extracted with four 20-ml portions of chloroform, and the combined extracts were evaporated to dryness on a water bath. The residue was dissolved in 20 ml of 1 *N* HCl and heated to boiling. After cooling, the solution was transferred quantitatively to a 100-ml volumetric flask and diluted to volume with water.

Two 10-ml aliquots were transferred to each of two 50-ml volumetric flasks (larger or smaller aliquots also were used, depending on the anticipated thebaine content). One flask was diluted to 50 ml with water and used for the blank. Two milliliters of 1% (w/v) sodium nitrite was added to the second flask, which was left standing for 15 min with occasional shaking. Then 5 ml of 10% (w/v) NH_4OH was added (changing the greenish-yellow color to orange), and the solution was diluted to 50 ml with water. This solution was stable for at least 20 min. The absorbance of the sample was measured against the blank, in 1-cm cells, at ~450 nm using a suitable spectrophotometer[3].

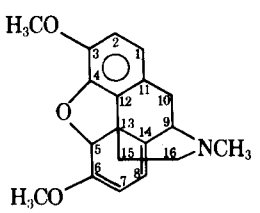
Preparation of Thebaine Calibration Curve—A stock standard solution was prepared by accurately weighing 100 mg of pure thebaine and transferring it to a 100-ml volumetric flask. The standard was dissolved and diluted to volume with methanol. A working standard solution was prepared by evaporating 3.0 ml of the stock standard solution to dryness on a water bath. The residue was dissolved in 20 ml of 1 *N* HCl and heated to boiling. After cooling, the solution was transferred quantitatively to a 100-ml volumetric flask and diluted to volume with water (1 ml = 0.03 mg of thebaine).

For the calibration curve, 5-, 10-, 15-, 20-, and 25-ml aliquots of the working standard solution were transferred to each of five 50-ml volumetric flasks. Two milliliters of 1% sodium nitrite was added to each flask. After the solutions stood for 15 min, with occasional shaking, the outlined procedure was followed using water for the blank. A standard calibration curve was prepared by plotting the milligrams of thebaine *versus* absorbance.

Table I—Elution of Thebaine from Alumina Columns with Different Solvent Systems

Solvent System	Percent Thebaine
Methanol–ammonium hydroxide (98:2)[a]	3.55, 3.56
Chloroform–calcium hydroxide[a]	3.55, 3.55
5% Acetic acid	
20 ml	3.58
40 ml	3.56
60 ml	3.58
80 ml	3.58
100 ml	3.56, 3.56, 3.57

[a] One hundred milliliters of solvent mixture.



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[1] The plant material used was the well-documented *Papaver bracteatum* Lindl.: Population Arya II, identified by Dr. P. G. Vincent (see Ref. 20).

[2] Merck 90, active acidic, Activity I.

[3] ELCO II colorimeter (Carl Zeiss) with an S49E filter (400–500 nm).

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Table II—Comparison of Thebaine Assays

Sample[a]	Colorimetric		GLC[b]	TLC–UV[c]
1 Kavadarci capsules	2.82	2.94	2.9	2.8
2 Kavadarci stems	0.39	0.38	0.4	0.39
3 Kavadarci roots	0.82	0.80	0.8	0.8
4 Berovo capsules	1.83	1.83	—	—
5 Berovo stems	0.21	0.20	—	—
6 Bitola capsules	2.64	2.72	—	—
7 Sv. Nikole capsules	3.54	3.50	3.5	3.44
8 Sv. Nikole stems	0.46	0.46	0.5	0.46
9 Vitacevo capsules	1.65	1.58	—	—
10 Vitacevo stems	0.06	0.07	—	—
11 Process, Phase I	0.20	0.21	0.2	0.2
12 Process, Phase II	2.20	2.25	—	—
13 Process, Phase III	0.54	0.52	—	—

[a] Samples 1–10 were obtained from plants grown in different regions of Yugoslavia. [b] From Ref. 7. [c] From Ref. 18.

DISCUSSION

Thebaine Degradation—Pure thebaine was boiled with varying normalities of hydrochloric acid from 1 to 15 min and was analyzed by TLC to determine the optimum conditions for degradation. It was found that 1 *N* HCl destroyed thebaine completely when the solution was brought to a boil.

Extraction—Two general extraction principles for thebaine isolation from *P. bracteatum* are found in the literature: (*a*) extraction with dilute acids as a salt (2, 4, 5, 7, 9, 13, 16), and (*b*) alkalinization of the wet plant material and extraction of thebaine with an organic solvent or a mixture of solvents as the free alkaloid (1, 3, 6, 8, 12, 19, 21–24). Three solvent systems were investigated: 5% acetic acid, methanol–ammonium hydroxide (98:2), and chloroform–calcium hydroxide (0.2 g of calcium oxide was added to the sample and water in the soaking step for sample preparation).

The procedure detailed here was followed using 100 mg of sample for each experiment. In the extraction step, 100 ml of each solvent system was used. As shown in Table I, the solvent systems quantitatively extracted thebaine from *P. bracteatum*. The 5% acetic acid extraction method was chosen to avoid an evaporation step before the alkaline extraction with chloroform.

It was found experimentally that 20 ml of 5% acetic acid was sufficient for complete extraction of thebaine from 100 mg of *P. bracteatum*. Thirty milliliters was specified in the procedure so that a sufficient volume was obtained.

At least three 20-ml volumes of chloroform were needed for the removal of the degraded thebaine from an alkaline medium. A fourth extraction is recommended, especially if an emulsion is formed.

pH Adjustment—Most published methods recommend a pH of 9 for the alkaloid extraction step. However, emulsions form less at pH > 11. Another reason for performing the extraction at pH > 11 is to prevent the interference of any phenolic alkaloids that may be present in the poppies. TLC evaluations of *P. bracteatum* revealed a second alkaloid, believed to be alpinigenine. A third alkaloid rarely was found. The TLC spots from these two alkaloids were removed and evaluated by the described procedure. Their eluates gave no color reaction with sodium nitrite solution.

Linearity—The absorption peak was a broad band peaking at ~450 nm. At this wavelength, the reaction followed Beer's law between 0 and 1.0 mg of thebaine. Examination of Tables I and II indicates that the method is reproducible.

Pigment Removal—Some pigments eluted from the alumina columns. An attempt was made to remove pigments by acid extraction with chloroform or ether (4, 5, 18) prior to the alkaline extraction with chloroform. The same results were obtained with and without the acid extract. Most of the pigments remained in the aqueous layer after the alkaline chloroform extraction in the recommended procedure.

Reaction Time for Color Development—At least 10 min was required for full color development with the 1% sodium nitrite solution. The orange color took 10 min to develop fully after the addition of the 10% NH_4OH and was stable for an additional 10 min. The absorbance readings must be made between 10 and 20 min after addition of the ammonium hydroxide.

RESULTS

Samples of *P. bracteatum* plants grown in different regions of Yugoslavia were assayed by the colorimetric method described here. Samples from various process phases of thebaine isolation also were examined (Table II). For process samples, aqueous solutions were extracted directly with chloroform at pH >11. If the thebaine had been processed with organic solvents, the solvent was removed by evaporation. The residue was dissolved in 5% acetic acid, and the remainder of the procedure was followed. For comparison, some samples were assayed by a GLC method (7) and by a TLC–UV procedure (18) (Table II).

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