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QUANTITATIVE DETERMINATION OF THEBAINE IN *PAPAVER BRACTEATUM* BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the quantitative analysis of thebaine from *Papaver bracteatum*, using a single high-pressure liquid chromatographic column. The procedure gives base-line separation of thebaine without the need for gradient elution equipment, and can be completed within 12 min. Thebaine isolated by this technique was shown to be pure, regardless of the age of plant or plant part from which it was obtained.

INTRODUCTION

In recent years, as a result of a general reduction in the production of legitimate codeine from *Papaver somniferum* (Opium poppy), concomitant with an increased demand throughout the world[1,2], other sources of raw materials for the production of this alkaloid have been sought. Although *Papaver bracteatum* has been reported to contain thebaine, isothebaine, orientalidine, and codeine, in addition to some 25 other alkaloids[3,4], there is some controversy as to whether the plants investigated were actually *P. bracteatum*, or natural hybrids of *P. bracteatum* with *P. orientale* or *P. pseudo-orientale*[5]. Thebaine, however, is consistently reported to be the major alkaloid of *P. bracteatum*[1,2,5,6], the other alkaloids being present only in trace amounts. Since thebaine can be chemically converted to codeine[6,7], *P. bracteatum* is currently regarded as the most promising solution to the codeine shortage[1,2,5,6], and many procedures for the extraction, purification, and quantitation of thebaine have been reported in recent years[3,5,6,8–21].

Gas–liquid chromatography (GLC) has been used routinely to separate and quantitate thebaine from *P. bracteatum* extracts[3,8–13]. However, none of these GLC procedures has established that the thebaine peak observed during routine analysis represents only pure thebaine. It was determined in this investigation that thebaine is decomposed into a mixture of products under the conditions routinely employed in GLC analyses. Since it was not possible to determine whether the GLC thebaine peak represented pure thebaine, or a mixture of phytoconstituents and/or their

decomposition products, a high-pressure liquid chromatographic (HPLC) procedure employing milder conditions was needed.

A number of HPLC separations of thebaine and other opium alkaloids have been previously reported[14–21]. However, these methods require pre-treatment of the extract to remove polar impurities (*e.g.*, ion-exchange chromatography), gradient elution equipment, and/or they do not produce base-line separation of thebaine from other constituents. A rapid, single column separation which overcomes the disadvantages of previous methods is described in this communication.

EXPERIMENTAL

Apparatus

A Perkin-Elmer Model 881 gas chromatograph equipped with a hydrogen flame ionization detector and a Sargent SR recorder were used for the GLC studies. A 6 ft. × 1/4 in. O.D. spiral glass chromatographic column was packed with 2.5% OV-17 on 100–120 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.).

Liquid chromatographic separations were conducted using a Waters Assoc. (Milford, Mass., U.S.A.) Model 6000 liquid chromatograph equipped with a Beckman 25 variable-wavelength UV spectrophotometer and recorder. The separations were carried out using a Waters Assoc. 30 × 4 mm I.D. μBondapak C_{18} column.

For thin-layer chromatographic (TLC) analyses, aluminum-backed, precoated silica gel GF_{254} plates (20 × 20 cm, 0.25 mm thick) or aluminum oxide F_{254}, type T (20 × 20 cm, 0.25 mm thick), both obtained from E. Merck (Darmstadt, G.F.R.) were used.

Reagents

All chemicals and solvents used in this investigation were reagent grade. Solvents for HPLC were redistilled in glass.

Standard thebaine (GLC pure) was obtained from S. B. Penick (Orange, N.J., U.S.A.).

Morphine and codeine were generated from morphine sulfate and codeine phosphate, respectively (Mallinckrodt, St. Louis, Mo., U.S.A.).

Isothebaine and orientalidine were provided by Professor E. Brochmann-Hanssen, University of California, San Francisco, Calif., U.S.A.

Papaver bracteatum alkaloid extracts

Dried, powdered (40 mesh) *P. bracteatum* straw (aboveground parts, excluding capsules; 1.0 g) was transferred to a 125-ml Erlenmeyer flask and 5% aqueous acetic acid (50 ml) was added. The flask was shaken for one hour by means of a rotary shaker, the mixture was filtered through a Büchner funnel, and the marc was washed with 5% aqueous acetic acid (10 ml). Following alkalinization of the filtrate with concentrated ammonium hydroxide (6 ml), the filtrate was extracted three times with chloroform (50 ml per extraction). The combined chloroform extracts were dried over anhydrous sodium sulfate, filtered, and the sodium sulfate was washed with chloroform (10–15 ml). The combined extracts were evaporated to dryness *in vacuo*, yielding a residue which was dissolved in an appropriate solvent and applied to a chromatographic column.

Gas–liquid chromatography

The GLC operating conditions employed were: injector temperature, 285°; detector temperature, 285°; oven temperature, 270°; flow-rate of carrier gas (helium), 48 ml/min; hydrogen pressure, 17.5 p.s.i.g.; air pressure, 50 p.s.i.g. In order to determine whether the thebaine peak represented pure thebaine, a stream splitter was installed at the elution end of the GLC column. Five repeated injections (10 μl) of *P. bracteatum* total alkaloid extract, containing approximately 6 μg thebaine per μl ethanol, were made, and the compounds represented by the two resulting peaks were collected separately.

The two resulting fractions were applied to a silica gel TLC plate and developed with toluene–acetone–ethanol–conc. ammonium hydroxide (20:20:3:1)[22]. Chromatograms were examined under both short- and long-wavelength UV light after development and then sprayed with 70% sulfuric acid in methanol. Compounds were detected by charring at 110° for 10 min. Two auxiliary TLC systems were also used *viz.*, silica gel with benzene–acetone–methanol (7:2:1)[23]; and aluminum oxide with benzene–ethanol (9:1)[24].

High-pressure liquid chromatography

The operating conditions for HPLC were: ambient temperature; flow-rate of eluting solvent, methanol–water containing 0.3% ammonium carbonate (4:1), 1 ml/min; wavelength of UV detector, 285 nm; recorder chart speed, 0.5 in./min. Standard solutions of thebaine, isothebaine, orientalidine, codeine, and morphine were injected onto the HPLC column and their retention times determined.

Two Beer's law standard curves were obtained by injecting different concentrations of standard thebaine onto the column in quadruplicate. The thebaine concentrations employed were 0.01, 0.02, 0.05, 0.075, and 0.10 μg/μl at 0.25 absorbance units full scale (a.u.f.s.) on the UV recorder, and 0.10, 0.20, 0.30, 0.40, 0.50, and 0.60 μg/μl at 0.5 a.u.f.s. (20 μl per injection). Peak areas were measured using the triangulation method.

For routine analyses of *P. bracteatum* alkaloids, the residue obtained from the chloroform extracts was dissolved in an accurately measured volume of chromatographic solvent and 20 μl of the resulting solution were injected in triplicate onto the HPLC column. To prevent damage to the column, it was routinely washed with redistilled water at the end of each day, until the pH of the effluent was 7. The column was then washed with methanol and stored.

In order to determine whether the thebaine peak represented pure thebaine, multiple injections of *P. bracteatum* alkaloid extract were made. The thebaine fractions were collected and combined, and a mass spectrum and UV spectrum were obtained. The isolated thebaine was also analyzed by TLC in all three chromatographic systems.

RESULTS AND DISCUSSION

Thebaine was apparently decomposed by the high temperature used during GLC, since no spot corresponding to thebaine could be observed on TLC chromatograms. The two collected GLC fractions were qualitatively similar, containing at least four compounds, but were quantitatively different. It was also observed that

the ratio of one compound to another in each fraction was a function of the GLC column temperature employed. If an oven temperature of 225° was used, only a single peak was observed on the recorder, and intact thebaine could be recovered. However, at this lower temperature, tailing of the thebaine peak was so severe as to preclude accurate quantitation. Decomposition of thebaine during GLC analysis was also noted by another research group[25].

Since thebaine is decomposed under the conditions routinely used in GLC analyses, it is impossible to determine whether the thebaine peak represents pure thebaine. Studies investigating the potential effects of chemicals on thebaine metabolism, therefore, could not be conducted using these GLC assay techniques. If, for example, a chemical being tested affected the metabolism of a plant constituent having the same retention time as the thebaine decomposition products, the effect of the chemical on thebaine metabolism would be obscured, since this phytoconstituent would be quantitated as thebaine. An HPLC procedure was developed to obviate this problem.

Under the conditions used for HPLC in this study, isothebaine, orientalidine, morphine, and codeine were all well separated from thebaine, giving the following retention times: isothebaine, 6.0 min; orientalidine, 6.0 min; morphine, 7.1 min; codeine, 7.9 min; and thebaine, 10.2 min. In addition, thebaine was well separated from all other compounds present in the total alkaloid extract of *P. bracteatum*. A typical chromatogram of this extract is shown in Fig. 1. Thebaine quantitation was also readily achieved by HPLC, since the detector response was linear for all concentrations employed. For the standard curve at 0.25 a.u.f.s. on the UV recorder, the slope was 3.02, the *y*-axis (peak area) intercept was +0.07, and the reliability was 0.999. At 0.5 a.u.f.s., the slope was 1.54, the *y*-axis intercept was −0.02, and the

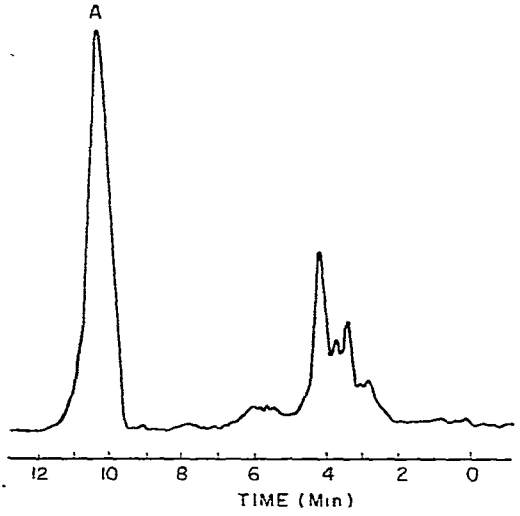

Fig. 1. Liquid chromatogram of *Papaver bracteatum* straw extract. Operating conditions: column, μBondapak C_{18}; mobile phase, methanol–water containing 0.3% ammonium carbonate (4:1); ambient temperature; flow-rate, 1.0 ml/min; detector, UV spectrophotometer (285 nm). Peak A = thebaine.

reliability was 0.999. The minimum and maximum amounts of thebaine which could be accurately quantitated were 0.05 μg (0.25 a.u.f.s.) and 12 μg (0.5 a.u.f.s.), respectively.

The mass spectrum and UV spectrum of thebaine, separated from a *P. bracteatum* total alkaloid extract by this HPLC column, were virtually superimposable with those of standard thebaine. In addition, TLC analyses showed a single spot in three different systems; the R_F corresponded in each case to that of standard thebaine. The R_F of thebaine in the primary TLC system was 0.49, in the auxiliary silica gel system it was 0.22, and in the aluminum oxide system it was 0.45. Further, thebaine isolated from roots and straw of plants ranging in age from 6 months to 2 years, as well as from immature or mature capsules, was found in each case to be pure by TLC in all three systems. Consequently, this HPLC technique can be applied to *P. bracteatum* alkaloid extracts regardless of plant age or plant part, even though these extracts contain different phytoconstituents.

The overall recovery of thebaine from the extraction and HPLC procedures was determined by adding 5.0 mg of reference thebaine to the exhausted marc of *P. bracteatum* straw (1.0 g). Re-extraction and analysis by HPLC yielded 4.63 mg thebaine, giving an overall recovery of 93%. Twelve replicate injections of alkaloid extract from *P. bracteatum* straw were also made to determine the precision of the HPLC quantitation (Table I). These data show that the reproducibility of the quantitative procedure is 98.1% within 99.7% confidence limits. Using this technique, thebaine concentrations as low as 0.0003% can be accurately quantitated if a 1.0 g plant sample (dry weight) is used for analysis.

TABLE I

REPLICATE ANALYSES OF *PAPAVER BRACTEATUM* TOTAL ALKALOID EXTRACT

Injection number	*Peak area (cm^2)*	*Thebaine weight (μg)*
1	12.65	8.21
2	11.73	7.61
3	12.19	7.91
4	12.05	7.82
5	11.94	7.75
6	12.33	8.00
7	12.04	7.81
8	12.22	7.93
9	12.09	7.85
10	12.32	8.00
11	12.18	7.91
12	12.56	8.15
Average	12.19 $\pm$ 0.08*	7.91 $\pm$ 0.05*

* Standard error of the mean.

The only problem regarding reproducibility of this technique involves column stability. Although μBondapak C_{18} consists of a monomolecular layer of octadecyltrichlorosilane, chemically bonded to silica via a hydrolytically stable ether linkage, repeated use of the column appeared to give rise to a small number of active sites on the silica. It was therefore necessary to saturate these active sites by making a daily

injection of 20 μg thebaine standard. In addition, injections of 5 μg thebaine standard were made until successive injections gave thebaine peak areas within 2% of each other or better; usually, two injections of thebaine were sufficient.

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