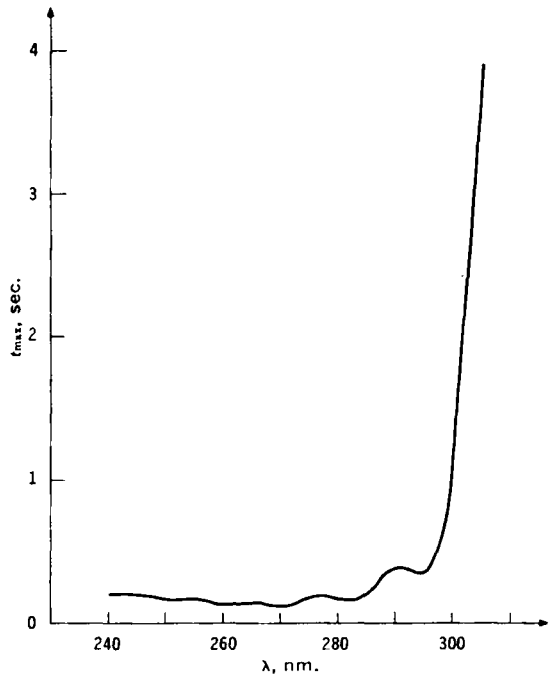

Figure 3—*Time needed to reach maximum previtamin D_2 production, with intensity of irradiation $\theta_0 = 10^{18}$ photons/cm.^{2} sec.*

isomers, when the previtamin has reached its maximum, are shown in Fig. 2. For the maximum of previtamin D_2 at the 295-nm. wavelength, there is a minimum of the three other isomers. The $N_2(t_{max})/N_3(t_{max})$, $N_2(t_{max})/N_1(t_{max})$, and $N_2(t_{max})/N_4(t_{max})$ ratios are, respectively, equal to 3.1, 10.2, and 47.5 at the 295-nm. wavelength and 1.8, 4.4, and 38.8 at the 280-nm. wavelength. This is an additional argument for seeking an irradiation source as rich as possible in UV radiation near to 295 nm.

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Quantitative Determination of Thebaine in Poppy Plants Using High Speed Liquid Chromatography

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Abstract □ A method for the quantitative determination of thebaine in poppy plants using high speed liquid chromatography is described. A nonionic polymeric adsorbent resin column cleanup of the sample is used to eliminate interferences from nonalkaloidal plant components. The separation is effected on a high efficiency adsorption column using *n*-hexane–chloroform–methanol–diethylamine (900:75:25:0.1) as the mobile phase. The column effluent is continuously monitored with a UV photometric detector (254 nm.). The thebaine fraction is well resolved from the isothebaine and orientalidine fractions. With 1-g. poppy samples, 0.01% thebaine is readily determined (detection limit of less than 25 ng.).

Keyphrases □ Poppy plants—analysis of thebaine using high speed liquid chromatography □ *Papaver bracteatum*—analysis of thebaine using high speed liquid chromatography □ Thebaine in poppy plants—analysis, high speed liquid chromatography □ Liquid chromatography, high speed—analysis, thebaine in poppy plants

A sensitive and rapid method was sought for the analysis of thebaine in small samples of poppy plants and parts thereof. The classical procedures of solvent extraction or column chromatography combined with gravimetric or titrimetric estimation of the isolated alkaloids were precluded (1, 2). Extraction methods for isolating the alkaloids from poppy plants and their subsequent separation for biosynthetic studies were previously described (3, 4). Adsorption chromatography on alumina followed by partition chromatography on

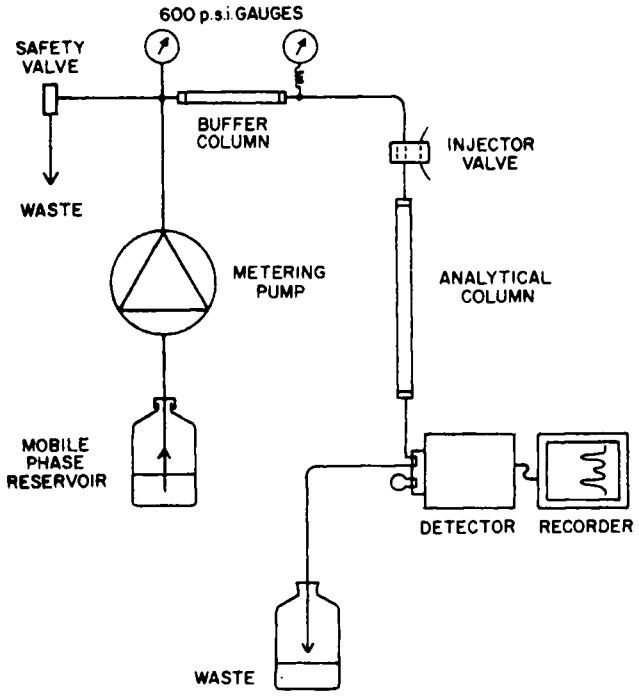

Figure 1—*Schematic diagram of the liquid chromatographic system.*

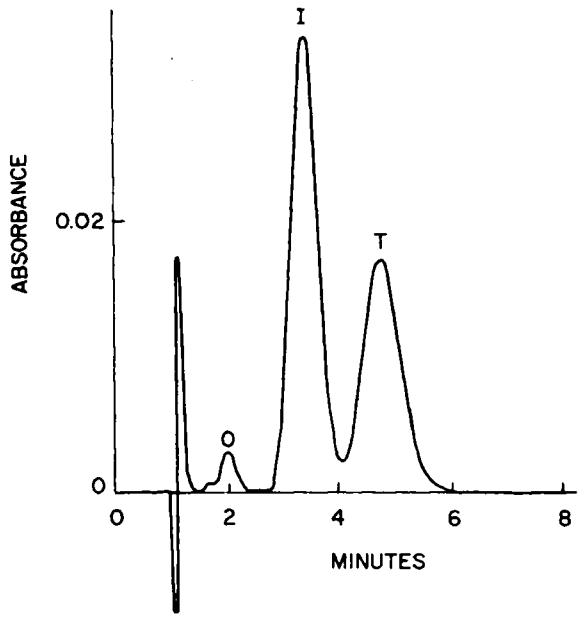

Figure 2—*Chromatogram of a synthetic mixture of 0.4 mcg. orientalidine (O), 1.0 mcg. isothebaine (I), and 1.0 mcg. thebaine (T).*

kieselguhr columns was used in the quantitative determination of the "secondary alkaloids" in opium (2). TLC on silica gel, followed by spectrophotometric measurement of the resolved alkaloids, was also used (5, 6). GLC was employed for the separation and quantification of opium alkaloids (7, 8), but problems due to adsorptive effects were encountered with GLC determination of the alkaloids (8). Other procedures include an ion-exchange separation (9) and an IR spectrophotometric determination (10). The latter procedure allows the simultaneous determination of narcotine, thebaine, and papaverine in opium by examining the IR spectrum of a carbon tetrachloride solution of the dried chloroform residue from an extraction of an acetic acid–water solution.

EXPERIMENTAL

Reagents—All reagents were analytical reagent grade, except diethylamine which was organic reagent grade. The nonionic polymeric adsorbent resin[1] (20–50 mesh) was washed before use with two, two, and four column volumes of acetone, methanol, and water, respectively. The strong cationic-exchange resin in the hydrogen form[2] (20–50 mesh), high efficiency adsorption packing[3] (37–50 μm.), TLC plates[4], and silicic acid[5] were used as received. A mixed solvent (Reagent A) consisting of chloroform–methanol (3:1) was used throughout the procedure.

Standards—Three thebaine standard solutions in the concentration range of 0.1–0.4 mg./ml. were prepared by dissolving thebaine alkaloid in Reagent A. The thebaine alkaloid was purified by classical purification procedures in these laboratories. It was shown to be single-component material by TLC, GLC, and high speed liquid chromatography (HSLC) and was found to be 99+% pure by nonaqueous acid–base titration. The standard solutions were freshly prepared each week and were stable when protected from light and solvent evaporation.

Equipment—A laboratory mill[6] was used to mill dried samples. A 1.4-cm. i.d. × 30-cm. glass column fitted with a Teflon stopcock was used for column chromatography in the cleanup procedure. A cotton plug was used for a bed support.

HSLC System—The system consisted of a mobile phase reservoir, a liquid metering pump[7], a pressure relief valve[8], a pulse dampening system, a 5-μl. sample injector valve[9], a 2.8-mm. i.d. × 300-mm. glass analytical column[10] containing a suitable packing[3], a UV (254-nm. wavelength) photometric detector[11], a recorder, various connectors and fittings, and 0.79-mm. (0.031-in.) i.d. Teflon tubing[10] arranged as shown in Fig. 1. The pulse dampening system was constructed from two 0–42.2-kg./cm.^{2} (0–600 p.s.i.) gauges[12] and a 2.8-mm. i.d. × 75-mm. column[10] packed with silicic acid. A 25-cm. coil of Teflon tubing was used to connect the second gauge to the fluid stream. At the beginning of each day, the coil was opened, drained, and reconnected to the system. The air column trapped in the coil served as an effective pulse suppressor to allow the detector to be operated at its most sensitive setting. The air slowly dissolved in the mobile phase, thus requiring its daily replacement.

Procedures—*Sampling and Extraction*—Fresh or frozen poppy roots, leaves, and/or stems were subdivided by chopping into about 0.5-cm. cubes (squares, if leaves). Dried roots, stems, leaves, capsules, or bracts were milled to approximately 1-mm. diameter particles. The subdivided sample was mixed to obtain a representative sample. Two portions of approximately 1 g. each were accurately weighed. A loss on drying at 105° (overnight) was determined on the first portion. The second portion was placed in a 250-ml. (8-oz.) blender jar, 100 ml. of water was added, and the pH was adjusted to 2 ± 0.2 with 10% (v/v) phosphoric acid solution. The sample was blended 4 × 30 sec. at high speed. Then the cutter blades were removed and the jar was placed in an ultrasonic bath. Filter pulp was added and the mixture was sonically agitated and simultaneously stirred with a motor-driven plastic paddle for 5 min. The slurry was filtered through paper with the aid of reduced pressure, and the filter cake was quantitatively washed with approximately 0.01 *N* phosphoric acid. The filter cake was saved and tested for thebaine content. The filtrate and washes were collected in a 400-ml. beaker, and the pH was adjusted to 9–10 with approximately 1 *N* sodium hydroxide.

Column Chromatography– A 10-ml. portion of prepared nonionic resin[1] was placed in the 1.4 × 30-cm. column. The sample was al-

[1] Amberlite XAD-2, Mallinckrodt Chemical Works, St. Louis, Mo.
[2] Dowex 50W-X2, Bio-Rad Laboratories, Richmond, Calif.
[3] Corasil II, Waters Associates, Inc., Framingham, Mass.
[4] ChromAR 7GF (250-μm. layer thickness), Mallinckrodt.
[5] SilicAR CC-7, 200–325 mesh, Mallinckrodt.
[6] Model No. ED-5, A. H. Thomas Co., Philadelphia, Pa.
[7] Milton Roy miniPump, Milton Roy Co., St. Petersburg, Fla.
[8] No. PRV500, Chromatronix, Inc., Berkeley, Calif.
[9] No. CSV-5, Chromatronix.
[10] Chromatronix.
[11] Model 200 with 1-mm. bore flow-through cell module, Chromatronix.
[12] Special No. 21540, Ametek/U. S. Gauge, Sellersville, Pa.

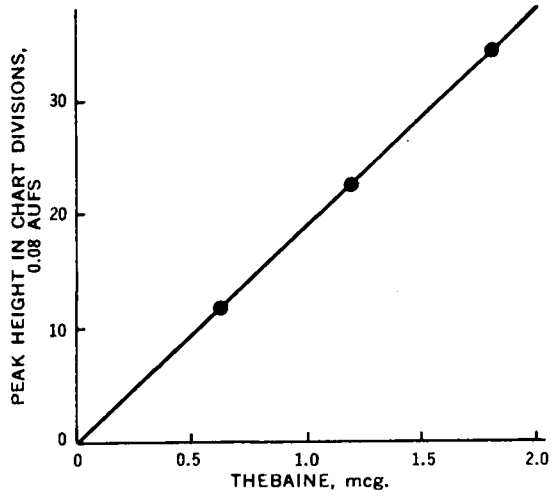

Figure 3—*Plot of peak height* versus *micrograms thebaine from chromatograms of thebaine standard solutions.*

lowed to pass through the column, adjusted to a flow rate of approximately 2 ml./min. The inside wall of the column was rinsed once with water, and then an additional 40 ml. of water was passed through the resin bed. The aqueous effluent was saved and checked for thebaine. A 200-ml. ⅋ 24/40 round-bottom flask was placed under the column. The inside wall of the column was washed with a few milliliters of methanol, and the alkaloids were eluted with 50 ml. of methanol. After approximately one-half of the methanol had gone through the column, the flow was stopped and the solvent voids were removed by running a stiff wire in and out of the resin bed. A cotton plug, to prevent the resin from floating, was placed on top of the bed; then the elution with the remainder of the methanol was resumed. Finally, 75 ml. of Reagent A was passed through the column. The resin bed was saved and checked for thebaine.

Sample Preparation for HSLC—The sample solution was evaporated to dryness using a rotary vacuum evaporator at 40–60°. Five milliliters of Reagent A was added to the sample residue in the flask. The flask was sealed with a polypropylene stopper and placed in an ultrasonic bath to extract the alkaloids from any insoluble residue. The solution was filtered through a medium-porosity, sintered-glass filter into a 25-ml. volumetric flask. The round-bottom flask was rinsed three times with 3-ml. portions of Reagent A, and each portion was filtered. Finally, the filter was washed twice with 3 ml. of Reagent A. Filtration was facilitated by positive pressure developed with a rubber squeeze bulb fitted on top of the filter funnel. The sample solution and washes were diluted to volume with Reagent A.

HSLC—The operating conditions for the analysis were as follows: (*a*) mobile phase composition, *n*-hexane–chloroform–methanol–diethylamine (900:75:25:0.1 v/v); (*b*) mobile phase flow rate, 1 ml./min. [approximate column head pressure of 2.8 kg./cm.^{2} (40 p.s.i.) was developed]; and (*c*) detector setting, 0.08 absorbance unit full scale (AUFS) for usual operation. The sample or standard solution was transferred to the sampling valve by means of a 1-ml. tuberculin syringe. Five microliters was then injected onto the column by manually actuating the sample injector valve. Peak heights of the thebaine peaks from sample and standard chromatograms were measured manually from the extrapolated baselines. Three standards were run each day to prepare a calibration curve. The thebaine concentrations of the samples were found from the calibration curve. A shared-time computer was used for constructing the calibration curve by finding the best slope (m) for a line of the equation $y = mx$, where y is the absorbance and x is micrograms of thebaine. Analytical results were calculated on the dried basis from the sample weights and loss on drying values.

TLC Procedure for Checking for Alkaloid Losses—*Filter Cakes*—The filter cake was placed in a beaker, about 50 ml. of water was added, and the pH was adjusted to approximately 3 with 10% (v/v) phosphoric acid solution. Five milliliters of cation-exchange resin[2] was added to the beaker. The mixture was then ultrasonically agitated and mechanically stirred for 5 min. The beaker was removed from the ultrasonic cleaner, and distilled water was added to fill the beaker. After the resin was allowed to settle, the supernate and suspended pulp were decanted. The water addition and decanting

Table I—Thebaine Assay Values of Several Iranian Poppy Plant Samples

Sample Number	Description	Weight Percent Thebaine (Dried Basis)
16B	Mature capsules	1.50, 1.41, 1.62, 1.42, 1.48
16C	Green stems	1.87, 1.83
16D	Bracts	1.42, 1.37
16E	Green capsules	1.23, 1.25
45B	Mature capsules	1.40, 1.48, 1.42

step was repeated at least three times. Most of the water was poured off, and then 25 ml. 1 *N* ammonium hydroxide in 70% methanol was added (11). The mixture was ultrasonically agitated 5 min. and then filtered through a cotton plug, fitted in a powder funnel, into a 100-ml. ⅋ 24/40 round-bottom flask. The resin was washed in the funnel three times with 5 ml. of 70% methanol. The filtrate and washes were evaporated to dryness on a rotary vacuum evaporator. The residue in the flask was extracted in 5 ml. of Reagent A with the aid of ultrasonic agitation, and 100 μl. was spotted (in 10-μl. increments) on a TLC plate.

The TLC conditions were as follows: (*a*) mobile phase, benzene–ethanol–ammonium hydroxide (90:15:1); (*b*) development, 10 cm., ascending; and (*c*) visualization, UV (254 nm.) and iodoplatinic acid spray reagent. Five microliters of a 1% thebaine solution was spotted adjacent to the sample spot for reference. The plate was developed and any thebaine found was noted. Any thebaine loss detected was cause to repeat the sample work-up using less sample.

Aqueous Effluents—The pH of the solution was adjusted to about 3 and treated as described previously beginning with the addition of the ion-exchange resin.

Posteluates—Alkaloid losses on the nonionic resin[1] were checked by passing another 50 ml. of Reagent A through the resin bed. The effluent was collected in a 100-ml. ⅋ 24/40 round-bottom flask and then evaporated to dryness on the evaporator. Five milliliters of Reagent A was added to the flask, and 100 μl. of the resulting solution was spotted (in 10-μl. increments) on a TLC plate.

RESULTS AND DISCUSSION

Figure 2 shows a chromatogram of a synthetic mixture of orientalidine, isothebaine, and thebaine. These three alkaloids are reported to occur in *Papaver bracteatum* and *P. orientale* (12, 13).

Figure 3 shows a plot of peak height *versus* micrograms of thebaine from chromatograms of thebaine standard solutions. For assay work, the system was calibrated each day because some variation in the thebaine retention volume was occasionally noted from day to day. Recalibration was also required when fresh mobile phase was used. Fresh mobile phase was prepared at least every 3 days because some loss in resolution was noted with 1-week-old mobile phase. However, the system was stable within a particular day. The peak heights for replicate injections of a standard sample throughout a 5-hr. period would nominally have an average variation of less than ±1% relative standard deviation (*RSD*).

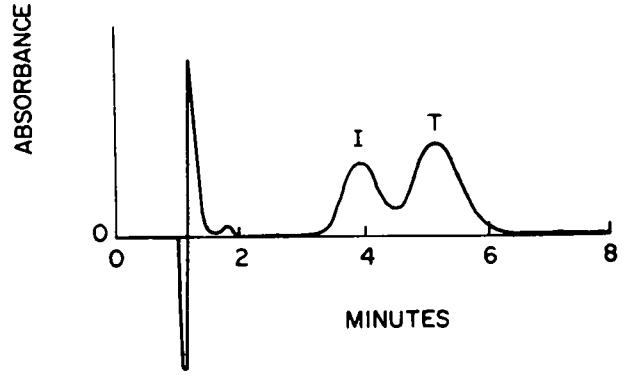

Figure 4—*Typical chromatogram of a poppy plant sample. Key: I, isothebaine; and T, thebaine.*

A chromatogram of a typical poppy plant sample is shown in Fig. 4. The retention volumes for the alkaloids are noted to be greater here than in Fig. 2. The chromatogram in Fig. 4 was run 2 months before the one in Fig. 2. The sample was 0.55 g. of capsules and assayed 0.39% thebaine on the dried basis (6.64% loss on drying).

Table I shows assay values for several Iranian poppy samples[13], tentatively identified as *P. bracteatum*. The coefficient of variation for the method was 5.7% with Sample 16B. It was felt that the precision obtained on this sample was limited by sampling error, since these data were obtained on a rather coarse (hand-ground) grind of the capsules. Duplicate assays on other samples ground finer by milling appeared to be in closer agreement.

TLC was used to check for thebaine losses as noted in the procedure. The sensitivity of a single TLC test was 1 mcg. of thebaine alkaloid (absolute). The cumulative thebaine loss from three TLC checks per sample could, therefore, not exceed 1.5%.

A procedure using a cation-exchange resin[2] (11) was tried and then abandoned. The resin is an efficient extractant for the alkaloids from the plant matrix. However, the resin extracted other plant matter, particularly from green stems and capsules, which eluted with the alkaloid fraction (1 *N* ammonia in 70% methanol was used to elute the alkaloids from the resin). Upon evaporation of the eluant, a large amount of residue remained from which the thebaine could not be quantitatively recovered. For such samples, the extraneous material was effectively removed with a postcolumn of a strong anionic-exchange resin in the chloride form[14]. For certain sample types, a gummy mass formed when the methanol–water phase was added to the cation-exchange resin[2] which hopelessly plugged the columns. However, the nonionic resin[1] procedure gave the least amount of extraneous residue and worked well with all sample types.

The nonionic polymeric adsorbent resin[1] system, as described here, has a capacity to handle about 50 mg. of thebaine. Losses due to incomplete extraction by, or elution from, the resin do occur with larger sample loads. The TLC monitoring of the sample cleanup procedure for different sample types easily determines if losses are occurring. The monitoring can be discontinued once an analyst determines the proper sample size to use.

[13] Obtained from Chemical Group Research and Development, Mallinckrodt.

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