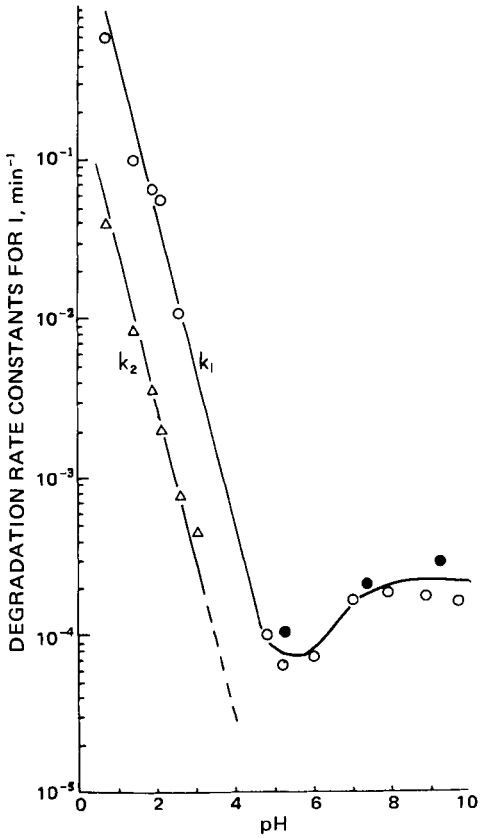

Figure 3—*Log* k–*pH profile for the degradation of I at 60.8°, where the concentration could be expressed as* $C = C_1e^{-k_1t} + C_2e^{-k_2t}$ *as a function of time, t, in minutes. The open symbols are previous data (1) and the closed symbols are additional data. The rate constants for monophasic degradation above pH 4 are taken as related to* k_1 *(see text) in contrast to the previous assignment (1).*

evaporated to dryness and reconstituted in 200 μl of chloroform. The solution was streaked on TLC sheets, developed for 15 cm in cyclohexane–acetone (9:1), and the sheets were analyzed by radiochromatogram scanner. No additional R_f values were observed other than those for GLC-identified products and a large amount of radioactivity at the origin. This result implies polar or polymeric degradation products that gave no pertinent signals under the GLC conditions, even after treatment of the methanolic extract of the origin with bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane[21].

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Direct Spectrophotometric Determination of Thebaine in Arya II Population Capsules of *Papaver bracteatum* Lindl

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Received February 9, 1976, from the *Department of Pharmaceutical Chemistry, College of Pharmacy, Tehran University, Tehran, Iran.*
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Abstract □ A simple spectrophotometric determination of thebaine based on the complexation reaction with bromcresol green was developed. The yellow complex was extracted with chloroform over the pH 1.5–4.5 range. The solution of the complex in chloroform showed the maximum absorption at 415 nm and obeyed Beer's law over the concentration range of 4.0–14.0 μg/ml. The molar absorptivity of the complex was 1.9460×10^4. The ratio of thebaine to bromcresol green in the complex was 1:1. The method was applied successfully to the direct determination of thebaine in the Arya II population capsules of *Papaver bracteatum* Lindl. The thebaine content of the dried capsules was 3.14%.

Keyphrases □ Thebaine—spectrophotometric analysis in Arya II population capsules of *Papaver bracteatum* □ *Papaver bracteatum*—Arya II population capsules, spectrophotometric analysis of thebaine □ Spectrophotometry—analysis, thebaine in Arya II population capsules of *Papaver bracteatum* □ Narcotics—thebaine, spectrophotometric analysis in Arya II population capsules of *Papaver bracteatum*

Most methods for the quantitative determination of thebaine (I) were based on chromatographic techniques (1–9). Other procedures include ion-exchange separation (10, 11), IR spectrophotometric determination (12, 13), and nonaqueous titration (14).

Several spectrophotometric methods have been reported (15–23). The UV spectrophotometric method (18) determines thebaine, when present alone, in completely pure solutions. Sulfuric acid methods (19–22) are specific but complicated and with low extinction values. A selective but

complicated method for the spectrophotometric determination of I also was reported (23).

In western Iran, a new population, Arya II, of *Papaver bracteatum* Lindl produced a high yield of I (24). The importance of I for the preparation of codeine and other medicinally important alkaloids prompted the use of this population as a natural source for I. Thus, a simple, rapid, and precise quantitative determination of I was needed to evaluate the plant samples.

This paper describes a new sensitive spectrophotometric method for I based on the complexation reaction between I and bromcresol green (II) followed by extraction with chloroform. Previously, II was investigated for the absorptiometric determination of small amounts of long-chain alkylamines and quaternary ammonium salts (25) in aqueous solution in a solvent extraction procedure that has the disadvantage of a blank depending on both the pH and concentration of excess reagent.

Prior separation of the alkaloid fraction, by chromatography or solvent extraction, is necessary for the determination by the reported methods. The present II method determines substances with a tertiary amine group, including thebaine, in aqueous acidic solutions without prior separation.

EXPERIMENTAL[1]

Reagents and Chemicals—All solutions were prepared from reagent grade[2] chemicals. Freshly prepared 10^{-4} *M* I, 10^{-4} *M* II aqueous solution, and pH 3 (BP standard) phthalate buffer were used.

Arya II Capsule Powder—The capsule powder[3] was sifted through a No. 42 sieve, and the remaining woody coarse particles were subjected to a current of air to free the fine powder.

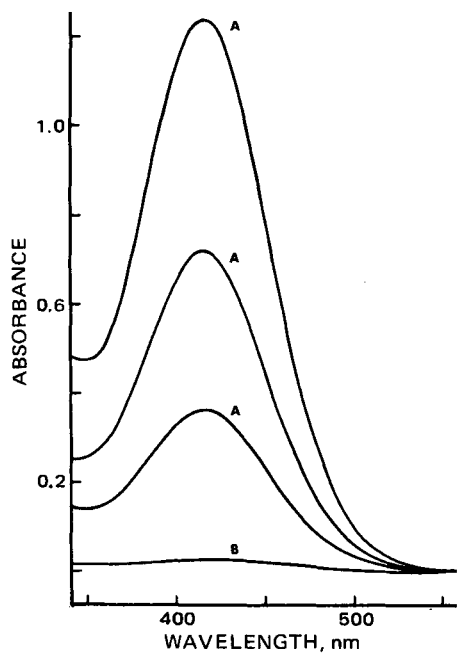

Figure 1—*Absorption spectra of chloroform solutions of various concentrations of I–II complex against the reagent blank (A) and of the reagent blank against chloroform (B).*

[1] A Beckman DB-GT spectrophotometer with 1.00-cm glass or quartz cells and a Beckman H3 type pH meter were used.

[2] Analar.

[3] Obtained from the Laboratory of Organic Chemistry, College of Pharmacy, Tehran University.

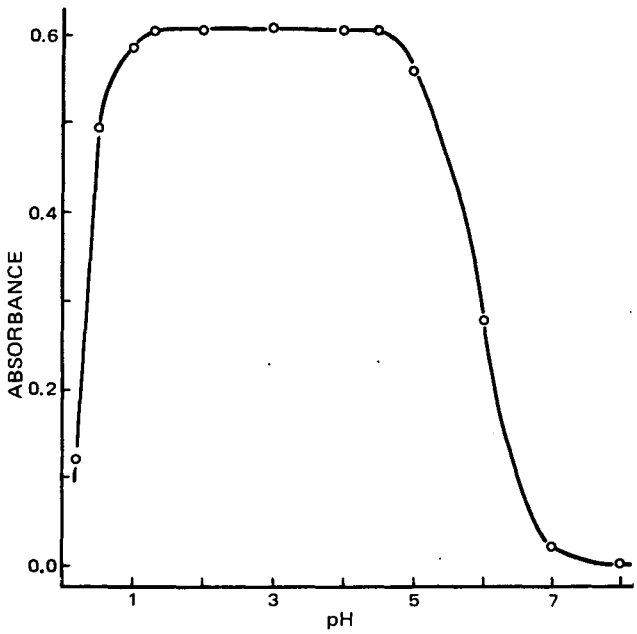

Figure 2—*Effect of pH on the complex formation and extraction. The concentration of I was 3 × 10⁻⁴ M.*

General Procedure—Pipet a sufficient quantity of I solution containing 40–140 μg of I into a 100-ml separator. Add 15 ml of 10^{-4} *M* II followed by a few drops of 0.1 *N* HCl or NaOH to obtain a yellow color. Then add 10 ml of pH 3 buffer and extract the yellow complex by vigorous shaking with 5, 3, and 2 ml of chloroform successively. Combine the extracts into a 10-ml volumetric flask, adjust the volume with chloroform, and measure the absorbance of the solution against a similarly prepared reagent blank at 415 nm.

The total operation requires about 10 min.

Direct Determination of Thebaine in Capsules—Digest accurately about 1.00 g of the Arya II capsule powder (fine powder, unsieved powder, and coarse particles) in 50 ml of 0.1 *N* HCl with intermittent shaking for 20 min. Filter the solution directly into a 250-ml volumetric flask. Repeat the digestion four times with 50 ml of 0.1 *N* HCl and filter immediately. Adjust the volume of the solution with distilled water to 250 ml and determine the I content as described in the general procedure.

Wash the clean glass apparatus with 0.1 *N* HCl followed by distilled water.

The following methods were employed to evaluate the proposed method.

Benzene Extraction Method (24)—The alkaloid was isolated by extraction with benzene and evaporation of the extract to dryness. The residue was dissolved in 0.1 *N* HCl, and the extinction at 285 nm (maximum) was measured using an $E_{1\%}$ (1 cm) value of 250.

In the present investigation, the proposed II method was employed, instead of extinction measurements at 285 nm, for the determination of thebaine content.

Sulfuric Acid Method (22)—The alkaloid was isolated by chloroform extraction and evaporation of the extract to dryness. The thebaine content of the residue was determined by using the color reaction with 29 *N* H_2SO_4.

RESULTS AND DISCUSSION

The yellow I–II complex solution in chloroform gave maximum absorption at 415 nm (Fig. 1). Complex formation and extraction were complete and quantitative between pH 1.5 and 4.5 (Fig. 2). The absorbance of the complex in chloroform was measured at selected times up to 1 week and was constant.

Composition of Complex—The composition of the complex was ascertained by the following methods.

Continuous Variation Method (26, 27)—The method was applied for the total constant concentration of the mixtures, [I + II], first at 10×10^{-4} *M* and then at 6×10^{-4} *M*; the complex was determined as described in the general procedure. Figure 3 shows that a maximum occurs at a 0.5 mole fraction of I, indicating thereby the formation of a 1:1 complex.

Mole Ratio Method—The method of Yoe and Jones (28) was applied

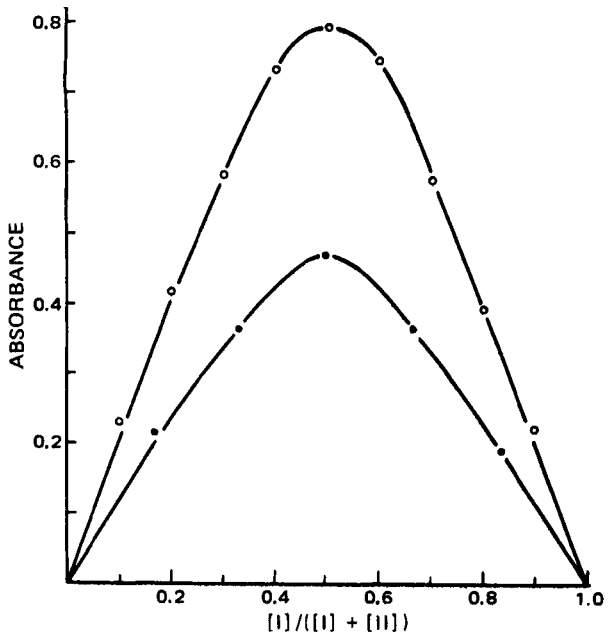

Figure 3—*Job's plot for the I–II system. Key (total concentration of I and II):* ○, *10 × 10⁻⁴ M; and* ●, *6 × 10⁻⁴ M.*

for a standard I solution at a fixed concentration of 4×10^{-4} *M* and different amounts of standard II solution of 10^{-4} *M*. The complex was determined as described. As shown in Fig. 4, the ratio of I to II in the complex was 1:1, but the required ratio for complete complexation and extraction was 1:3.

Beer's Law, Range, and Precision—The optimum concentration range for the measurements, which conforms to Beer's law, at 415 nm and a 1.00-cm optical path was about 4.0–14.0 μg/ml; the molar absorptivity of the complex solution in chloroform was 1.9460×10^4. The relative standard deviation of the calculated absorptivities in the optimum concentration range was 0.9%; at concentrations below 5.0 μg/ml, it was 2.2%.

Compound II Reactions—Acid dyes react with amines or quaternary ammonium salts to form colored compounds (29, 30). Compound II was used for the determination of small amounts of long-chain tertiary alkylamines and quaternary ammonium salts (25).

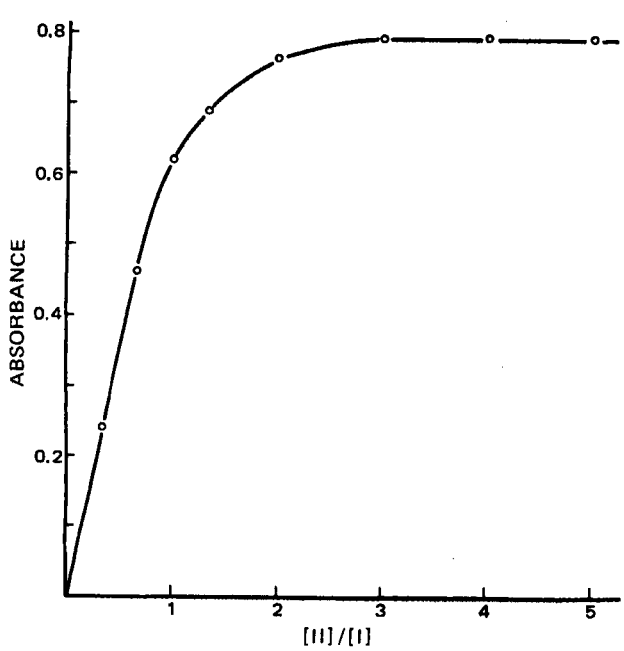

Figure 4—*Mole ratio plot. The final concentration of I was* 4×10^{-4} M.

Table I—Thebaine Content of the Arya II Population Capsules of *P. bracteatum* Lindl

Powder Portion[b]	Direct Determination[a] % Thebaine, Mean $\bar{X}$	Direct Determination[a] *RSD*, %	Indirect Determination[a] % Thebaine, Mean $\bar{X}$	Indirect Determination[a] *RSD*, %
Fine powder	4.17	1.13	3.70	1.43
Unsieved powder	3.14	1.10	2.71	1.29
Coarse particles	1.75	1.14	1.57	1.38

[a] Each determination was repeated six times on each portion. [b] The sample was collected during June 1974.

In an acidic medium, most organic compounds with a tertiary amine group react with II, forming yellow complexes extractable with chloroform. Antihistaminic agents and most alkaloids show selective positive reactions, and the proposed method was found to be suitable for their spectrophotometric determination. Further investigations on the validity of II for the determination of medicinally active substances in pharmaceutical preparations are currently being undertaken and will be the subject of future reports.

Determination of Thebaine Content of Arya II Population Capsules—Since thebaine is the only alkaloidal constituent of the Arya II population of *P. bracteatum* (24), it can be measured directly as described. The I content of different portions of the powdered capsules was determined directly after digestion with 0.1 *N* HCl and indirectly after benzene extraction (24), both with the II method (Table I). Moreover, the I content of each sample was determined by the sulfuric acid method (22), and identical results to the II method were obtained in each case.

In contrast to the sulfuric acid method (22), this method shows no interference from the other contents of the capsules. The indirect determination of the alkaloid based on benzene extraction gives a lower recovery (86–89%) of the alkaloid (Table I). This method, compared with the previously reported methods, was easier and more precise.

The presence of a high percentage of thebaine (1.75%) in the coarse particles, which are commonly discarded, necessitate their use as a source for thebaine production.

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Inhibition by Paromomycin of R-Factor Transfer of Tetracycline Resistance between *Escherichia coli* and *Salmonella pullorum*

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Received October 12, 1976, from the *Farmitalia Research Laboratories, 20014 Nerviano, Milan, Italy.* Accepted for publication February 2, 1977.

Abstract □ *In vitro* assays showed that low concentrations (5–10 and 20 μg/ml) of the antimicrobial paromomycin sulfate are able to block or diminish significantly the transfer of the tetracycline resistance R-factor between *Escherichia coli* and *Salmonella pullorum*. This observation is important because it offers the possibility of preventing the formation of tetracycline-resistant pathogens, a limiting factor of tetracycline use in both human and veterinary medicine.

Keyphrases □ Paromomycin—effect on R-factor transfer of tetracycline resistance from *Escherichia coli* to *Salmonella pullorum* □ Tetracycline resistance—R-factor transfer from *Escherichia coli* to *Salmonella pullorum*, effect of paromomycin □ Bacterial resistance—to tetracycline, R-factor transfer from *Escherichia coli* to *Salmonella pullorum*, effect of paromomycin □ Antibacterials—effect of paromomycin on R-factor transfer of tetracycline resistance from *Escherichia coli* to *Salmonella pullorum* □ Antimicrobials—effect of paromomycin on R-factor transfer of tetracycline resistance from *Escherichia coli* to *Salmonella pullorum*

Previously (1), it was demonstrated that *in vitro* pretreatment of *Escherichia coli* with low doses of tetracycline inhibits the production of mutants resistant to paromomycin sulfate (I), an oligosaccharide antimicrobial with a broad antibacterial spectrum including Gram-positive, Gram-negative, and acid-fast bacteria (2–4). Compound I also inhibits some protozoa (5, 6).

The effect of I on the development of tetracycline-resistant strains was studied by evaluating its action on the R-factor transfer capacity between a strain of *E. coli* (donor) and a strain of *Salmonella pullorum* (recipient) (7).

Salmonellosis is an increasing health hazard (8–11). The spread of drug-resistant pathogenic *Salmonella* has encouraged investigation of methods to reduce this phenomenon, which affects both human and animal bacterial flora.

EXPERIMENTAL

Bacterial Strains—*E. coli* (FI 3632), tetracycline resistant and rifamide sensitive, and *S. pullorum* (FI 741), tetracycline sensitive and rifamide resistant, were used. The *Salmonella* strain was used because only mutants with acquired resistance to tetracycline develop in subcultures on a solid medium containing 30 μg of tetracycline/ml plus 40 μg of rifamide/ml.

In these transfer assays, the two strains can be immediately distinguished on a solid medium containing lactose and a pH indicator by the formation of acidifying (*E. coli*) or nonacidifying (*S. pullorum*) colonies. The usual cultural, biochemical, and serological tests were employed to identify the parent and revertant strains.

Assay Media—Medium 1 was liquid tryptose broth[1]. Medium 2 (solid) contained tryptose agar[1], 1% lactose, and 0.01% bromthymol blue (Wurtz medium) with and without tetracycline[2] (30 μg/ml), rifamide[3] (40 μg/ml), paromomycin[4] (30 μg/ml), or tetracycline (30 μg/ml) plus rifamide (40 μg/ml). Medium 3 was TSI medium[1], and Medium 4 was SIM medium[1].

Medium 1 was used for the conjugation assays and the determination of the minimum inhibitory concentrations (MIC) of the three antimicrobials. Medium 2 served as a control for the conjugation experiments and for the sensitivity tests following the Kirby–Bauer method. The medicated media were employed for checking the drug resistance of the parent and revertant strains. Media 3 and 4 were employed for an initial differentiation between *E. coli* and *S. pullorum* colonies.

Reagents—Sensitivity disks were prepared according to the "Code of Federal Regulations" (12) and contained 30 μg of tetracycline, 40 μg of rifamide, or 30 μg of I. The somatic serodiagnostic antiserum of *E. coli* was obtained by rabbit immunization as described by Kauffmann (13), and the somatic serodiagnostic antiserum of *S. pullorum* was purchased[1].

Antibiotic Sensitivity Test—This test was performed in tryptose broth by determining the MIC values, in solid Medium 2 according to the Kirby–Bauer method, and by evaluating the bacterial growth in Medium 2 containing 30 μg of the single antimicrobial/ml.

Conjugation Assays in Presence of I—*Single Strains with I and 2–4 hr of Contact Time*—Compound I (final concentrations of 5, 10, and 20 μg/ml) was added to tryptose broth cultures of the single strains after 2 hr of incubation at 37°. After further incubation (2 and 4 hr), the cultures were washed (three times by centrifugation) to remove I and the pellets were resuspended and brought to volume with sterile tryptose broth.

Under these conditions, the drug concentration remaining in the cultures was lower than the minimum assayable microbiologically (0.05 μg/ml) by the agar plate method (12). Broth cultures of the two test strains grown in the absence of I were treated identically. The final bacterial suspensions thus obtained were mixed in a chessboard scheme according to the different concentrations of I with which the bacteria had previously been in contact.

[1] Difco Laboratories, Detroit, Mich.
[2] Lot 241/5, Farmitalia Research Laboratories, Milan, Italy.
[3] Lot 011, Gruppo Lepetit, spa, Milan, Italy.
[4] Aminosidine, lot 501.2, Farmitalia Research Laboratories, Milan, Italy.