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Quantitative Determination of Hyoscyamine and Scopolamine by Direct Photodensitometry of Thin-Layer Chromatograms

B. L. WU CHU, E. S. MIKA, M. J. SOLOMON, and F. A. CRANE

Abstract □ A rapid and simple method for the quantitative evaluation of thin-layer chromatograms of hyoscyamine and scopolamine by direct photodensitometry was investigated. Excellent separation with discrete spots for the two alkaloids was quickly obtained using absolute methanol–ammonia T.S. (200:1 v/v) as the developing solvent. Spots were made visible by spraying with Munier and Macheboeuf's modification of Dragendorff's reagent. Linear standard curves were obtained for each alkaloid when the integrated function of the size and density of the spot was correlated with micrograms of alkaloid applied. The upper and lower limits of the standard curves were 4 to 65 mcg. for hyoscyamine, and 7 to 60 mcg. for scopolamine. The optimal range for both alkaloids was approximately 20 to 30 mcg. Pure alkaloids and plant extracts were determined by photodensitometric and spectrophotometric methods, and results were compared.

Keyphrases □ Hyoscyamine, scopolamine—separation, determination □ TLC—separation □ Dragendorff's reagent—color development □ Photodensitometry, direct—quantitative analysis

The qualitative resolution of complex mixtures by thin-layer chromatography (TLC), introduced by Stahl (1) in 1956, has been extensively investigated. The quantitation of these resolved compounds has been less extensively studied. Quantitative TLC is in essence based upon either elution of the compounds contained in the spot or measurement directly on the plate.

The quantitative method of relating spot size to the quantity of compound on thin-layer chromatograms was thoroughly investigated by Purdy and Truter (2, 3). They explored three general methods of analysis based upon spot size and found a linear relationship between the square root of the area and the logarithm of the weight.

Privett and Blank (4, 5) described a method for the quantitation of lipids separated by TLC by applying direct densitometry to thin-layer chromatograms sprayed with a saturated solution of potassium dichromate in 80% (by weight) sulfuric acid. The plate was subsequently heated at 180° for 25 min. in order to char the spots. The area under the densitometer curves was directly proportional to the amount of sample in the spot. The direct scanning procedures for TLC plates have been evaluated critically by Klaus (6). Optical densitometry of charred spots has been extensively applied to determinations of lipids and glycerides but does not appear to have been used widely before with the studies of alkaloids. Genest *et al.* (7, 8) reported quantitative TLC data by direct densitometry on certain alkaloids of morning glory seeds and opium. Shellard and Alam (9) described a TLC densitometric method for the quantitation of some *Mitragyna* oxindole alkaloids.

The present work was undertaken to develop the technique of direct densitometric measurement of individual TLC spots of hyoscyamine and scopolamine as free bases. This densitometric method of area measurement is simple to perform and compares favorably with spectrophotometric techniques. Furthermore, in using a photoelectric device, the process of measurement is rapid and convenient.

EXPERIMENTAL

Equipment—Chromoscan recording and integrating densitometer equipped with TLC attachment (Joyce, Loebl and Company, Ltd., Gateshead-on-Tyne, Great Britain), spectrophotometer (Beckman model DU), pH meter (Beckman zeromatic), centrifuge, flash evaporator, adjustable TLC applicator (model SII Desaga/Brinkmann Instruments, Inc.), standard glass carrier plates (20 × 20 cm.), glass developing tanks (Desaga) lined with solvent-saturated filter paper, lambda pipets.

Materials—*Hyoscyamus niger* L., field-grown, oven-dried at 50°, ground to 40 mesh, served as the standard plant powder. All chemicals used were analytical reagent grade. Hyoscyamine (New York Quinine and Chemical Works, Inc.) and scopolamine (Aldrich Chemical Co.) were used. Each alkaloid standard produced only one spot on a chromatogram.

Operating Conditions for Densitometer—Chromoscan: filter 490 mμ, aperture 10 × 0.5 mm., cam A, gain 5, optical wedge 0–0.5 o.d.; thin-layer attachment: filter 490 mμ, aperture 5 × 0.5 mm., specimen expansion ratio 1:2. Light source for both units was a 12 v., 100 w. standard tungsten projection lamp. Both units were operated by reflectance.

Preparation of the Plates—Silica Gel G was purified by refluxing with anhydrous methanol for 8 hr. in a continuous extraction apparatus. Thirty-three grams of purified Silica Gel G was mixed with 67 ml. of distilled water and shaken vigorously for 45 sec. in a glass-stoppered conical flask. The slurry was spread on 20 × 20-cm. standard glass carrier plates to a thickness of 500μ. The coated

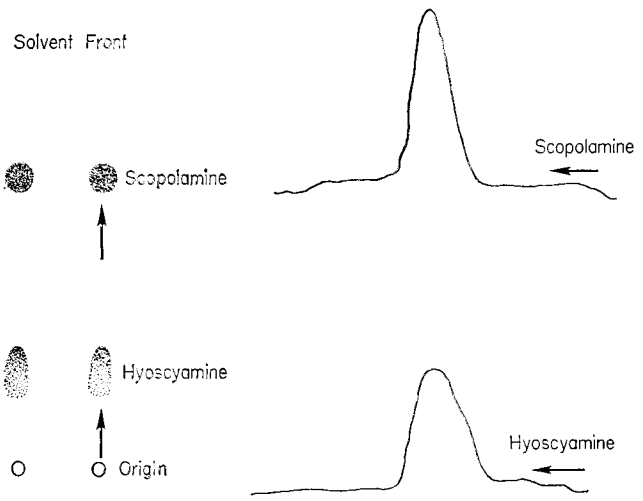

Figure 1—(**a**) *Tracing of a chromatogram of hyoscyamine and scopolamine;* (**b**) *tracing of densitogram of hyoscyamine and scopolamine.*

plates were air-dried at room temperature for about 15 min., followed by further drying at 105° for 30 min. The activated plates were stored in a desiccator which contained activated alumina.

Developing Solvent—The developing solvent was absolute methanol–ammonia T.S. (200:1 v/v). The advantage of this solvent system is that it gives discrete spots which are 6–7 cm. apart in 55–60 min. with a 16-cm. solvent front.

Spray Reagent—Munier and Macheboeuf's (M.M.) modification of Dragendorff's reagent (10) was used.

Application of Samples and Development of Chromatogram—Samples were applied in chloroformic solution approximately 2.5 cm. from the edge of the plate. Calibrated lambda pipets were used for initial application, and the resultant diameter of the spot obtained was approximately 5 mm. Plates were developed by ascending chromatography through a distance of 16 cm., dried at room temperature, and sprayed with modified Dragendorff's reagent (10). Standard samples of hyoscyamine and scopolamine free bases were always applied and chromatographed on the same plate to serve as reference standards.

Direct Photodensitometry—The developed chromatogram was scanned face-up in the same direction as the solvent flow. Each spot was scanned individually. The sample beam in each determination covered the entire width of a given spot. Total absorbance of each spot which represented the total area under the density curve was converted quantitatively by the automatic digital integrator. Background correction was made according to the method of Bush (11).

Spectrophotometry—Freeman's method (12) was employed except that the spectrophotometer was operated at 560 mμ rather than 540 mμ and 0.7 ml. of 10% aqueous tetraethylammonium hydroxide was used instead of 0.3 ml. of a 25% solution.

Preparation of Standard Curves—*Densitometry*—Six separate chloroformic solutions of hyoscyamine and scopolamine were prepared. Linear standard curves were obtained for both hyoscyamine and scopolamine when the integrated function of the size and density of the spot was correlated with micrograms of alkaloids

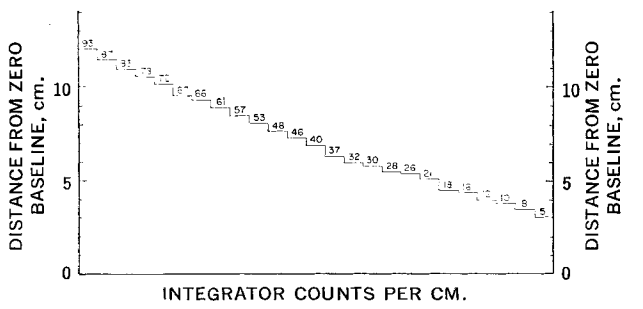

Figure 2—*Calibration curve for background correction in densitometry.*

applied. Blind unknowns containing weighed amounts of hyoscyamine and scopolamine in chloroform were used to substantiate the reliability of the standard curves.

Spectrophotometry—Linear standard curves were obtained for hyoscyamine, scopolamine, and the two as a 1:1 mixture. Blind unknowns substantiated the reliability of the standard curves.

Quantitation of the Alkaloids of a Plant Powder—Approximately 4 g. of hyoscyamus powder was placed in a 50-ml. glass-stoppered centrifuge tube, followed by the addition of 2 ml. ethyl alcohol (95%) and 1.5 ml. of 10% ammonium hydroxide. The tube was shaken for approximately 5 min. and refrigerated for 1 hr. Ten milliliters of *n*-butanol–benzene solution (1:1) was then added. After shaking vigorously for 2 min., the material was centrifuged for 3 min. at 1500 r.p.m. The upper layer which contained the alkaloids was then introduced into a 125-ml. separator with a capillary pipet without disturbing the solid residue at the bottom of the centrifuge tube. This was repeated four times with 7.5 ml. of *n*-butanol–benzene. In each case the residue was resuspended in the solvent with a glass rod and vigorously shaken for 1 min. The combined extracts were washed with 7.5 ml. of 0.5 *N* sulfuric acid, the mixture was allowed to separate, and the lower acidic layer was retained. This procedure was repeated three times using 5 ml. of 0.5 *N* sulfuric acid and finally 5 ml. of distilled water.

The combined acidic aqueous phase was titrated with concentrated ammonium hydroxide to a pH of 10.3 and was transferred into a 125-ml. separatory funnel containing 5 ml. chloroform. This extraction with chloroform was repeated five times or until 2–3 drops of the last extract gave a negative Vitali-Morin test (13). The combined chloroform extracts were washed with 5 ml. distilled water and the chloroform layer was placed in a dry 200-ml. round-bottom flask. The aqueous layer was agitated with 2 ml. chloroform and the chloroform added to the combined chloroform extract. This was evaporated to dryness on a flash evaporator. The dry residue was dissolved in chloroform and placed in a 10-ml. volumetric flask.

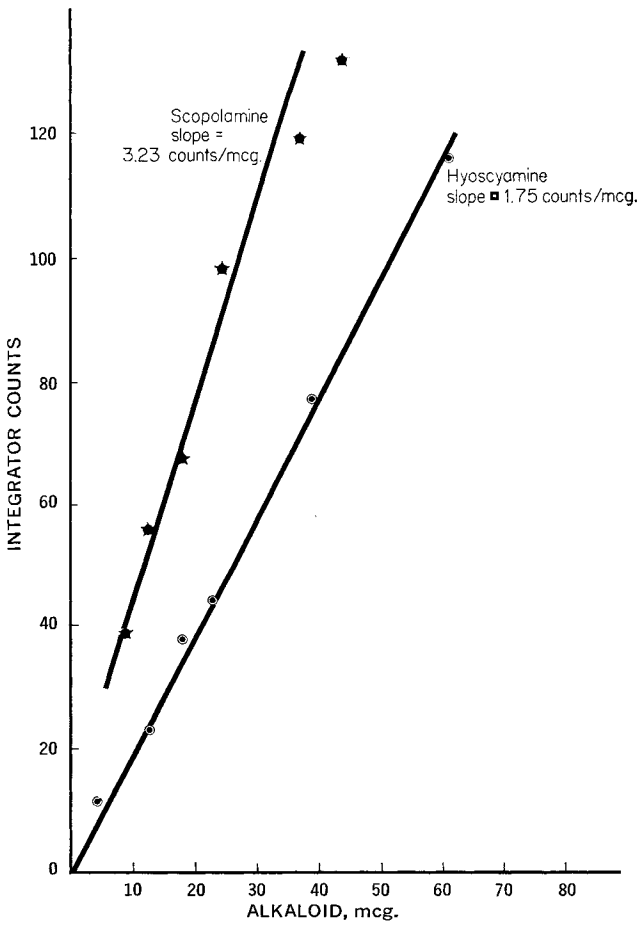

Figure 3—*Standard curves for hyoscyamine and scopolamine determined by densitometry.*

Table I—Simultaneous TLC-Densitometric Determination of Hyoscyamine and Scopolamine as a Mixture

Sample[a]	Hyosycamine			Scopolamine		
	Actual, mg.	Determined, mg.	Recovery, %	Actual, mg.	Determined, mg.	Recovery, %
1	19.26	16.25	84.38	13.13	13.17	100.30
2	14.80	13.68	92.43	18.26	18.91	103.56
3	19.61	16.25	82.87	20.29	20.92	103.10
4	22.84	20.24	88.62	26.26	26.19	99.74

[a] Each sample was weighed separately.

Aliquots of the 10-ml. extract were quantitated by direct photodensitometry and by spectrophotometry.

RESULTS AND DISCUSSION

Precision of the densitometer was estimated by scanning the same alkaloid spot 10 times successively within 30 min. after spraying. This was carried out throughout the entire investigation. The average percentage error was 6.06%, ranging from 2.11 to 8.11%, as determined on various occasions.

Figure 1*a* is a tracing of a finished chromatogram of two hyoscyamine and scopolamine samples. Figure 1*b* is a tracing of the densitogram curves of the two spots made by the Chromoscan.

The stepwise curve (Fig. 2) shows digital integrator values per centimeter length of the graph at different baseline levels. It was used for background correction of all density curves. Each value shown was the average of 10 determinations.

Standard curves for hyoscyamine and scopolamine obtained by direct densitometry are shown in Fig. 3. Since the measurements produced a straight standard curve, resort to logarithms and square roots of areas is not necessary. Each curve is the average of three independent determinations. Each of the six values was obtained from separate solutions using weighed quantities of alkaloids. The upper and lower limits of the standard curves were 4 and 65 mcg. for hyoscyamine, and 7 and 60 mcg. for scopolamine. The optimal range for both alkaloids was approximately 20 to 30 mcg. The slopes of the lines remained constant, 1.75 integrator counts per microgram for hyoscyamine while the corresponding value for scopolamine was 3.23 integrator counts per microgram. The small variation in the slopes obtained may be attributed to the intensity of stray light on the photomultiplier, the ambient temperature, spot shape, and variations in layer thickness.

Hyoscyamine produced an ellipitcal spot on the resulting chromatogram with R_f value of 0.097, whereas the scopolamine spot with R_f value of 0.52 was isodiametric. The difference in slope values of the standard curves of the two alkaloids might be attributed, at least in part, to the marked difference in the spot shape. The R_f value was the average from 10 chromatograms, each consisting of six spots.

Although many methods of thin-layer and paper chromatography (14–16) have been applied to the separation of tropane alkaloids, few reports have described a rapid and simple method. In the course of the present study of the growth, development, and alkaloid content of *Hyoscyamus niger* L., the new solvent system, absolute methanol–ammonia T.S. (200:1 v/v), gives a quick simple method for obtaining an excellent separation with discrete spots of the two major tropane alkaloids, hyoscyamine and scopolamine. A substance positive to Dragendorff's reagent using the plant extract appeared and stayed at the origin. This did not contaminate hyoscyamine, the nearest spot. The identity of this substance was not ascertained.

Chloroform was found to be the most satisfactory solvent. Uniform spots with minimum dispersion of solutes were produced. Different developing solvent systems were tried in order to make the hyoscyamine spot isodiametric. The relatively low percentage recovery of hyoscyamine (Table I) might be due to the greater error introduced by the elliptical shape of its spot on the chromatogram. A possibility exists that the elliptical shape resulted from imperfect dehydration of hyoscyamine. However, highly purified hyoscyamine dissolved in purified chloroform behaved in a similar manner, thus implicating the developing system.

Spectrophotometric data using Freeman's method (12) for the four mixed samples of hyoscyamine and scopolamine were compared with TLC-densitometric method (Table II). Percentage recovery of total alkaloids by spectrophotometry in which standard alkaloids were used was 97 to 98% while the TLC-densitometry recovery was from 91 to 99% when the hyoscyamine and scopolamine determinations were combined. In Table III, spectrophotometric determination of standard hyoscyamus powder is compared with TLC densitometric determination. If the spectrophotometric values obtained by use of standard hyoscyamus powder are used as reference standard, the percentage recovery of TLC-densitometric values would be 100 ± 5%. This suggests that the spectrophotometric determination of total alkaloids might well be used as the reference standard rather than the "actual amount of plant sample weighed" in which the alkaloid content may vary.

The present study substantiates the importance of each of the following factors as reported by other authors (9, 17–24) in their discussion of TLC-densitometry:

1. Type and quality of support, thus calling for a homogeneous adsorbent.
2. Adsorbent layer thickness, requiring a very uniformly prepared plate.
3. Particle size and activity of the adsorbent.
4. Uniform application of spots from solutions the concentrations of which are not very different.
5. A very small initial spot size.
6. Position of origin and distance of development.
7. Use of a calibrated micropipet.
8. Constant vapor saturation in the developing chamber.
9. Solvents that give a sharp and distinct separation of the substances being determined.
10. Working temperature.
11. Spray reagents that give maximum color contrast between spot and background.
12. Sensitivity of the color reaction, time of color development, time of attainment of maximum color intensity, and stability of spot color.
13. Application of reagent in a uniform spray.
14. Method of standardization and running the standard at the same time that the unknown is run.

Table II—Comparison of Spectrophotometric Determination with TLC-Densitometric Determination of Mixtures of Hyoscyamine and Scopolamine

Sample[a]	Actual, mg.	Spectrophotometry		TLC-Densitometry			
		Total Alkaloids, mg.	Recovery, %	Hyoscyamine, mg.	Scopolamine, mg.	Total Alkaloids, mg.	Recovery, %
1	32.39	31.8	98.18	16.25	13.17	29.42	90.83
2	33.06	32.5	98.31	13.68	18.91	32.59	98.58
3	39.90	39.2	98.25	16.25	20.92	37.17	93.16
4	49.10	47.5	96.74	20.24	26.19	46.43	94.56

[a] Each sample was weighed separately.

Table III—Comparison of Spectrophotometric Determination with TLC-Densitometric Determination of Standard Hyoscyamus Powder

Powder Plant Sample	Amount Weighed, g.	Spectrophotometry: Total Alkaloids, mg.	Spectrophotometry: % of Sample	TLC-Densitometry: Hyoscyamine, mg.	TLC-Densitometry: Scopolamine, mg.	TLC-Densitometry: Total Alkaloids, mg.	TLC-Densitometry: % of Sample	% Recovery if Spectrophotometric Determination is Used as Reference Standard
1	3.73	0.658	0.018	0.385	0.303	0.688	0.018	104.5
2	3.23	0.526	0.016	0.257	0.279	0.536	0.017	101.9
3	3.15	0.488	0.015	0.274	0.241	0.515	0.016	105.5
4	3.56	0.545	0.015	0.313	0.229	0.542	0.015	99.4

15. A constant slope value of linear relationship between spot area and quantity of applied compound.

This work has controlled the variables stated above and has reaffirmed the validity of the photodensitometric method as a simple and efficient technique for quantitative evaluation of thin-layer chromatographic results.

SUMMARY

A thin-layer chromatographic technique using Silica Gel G as the adsorbent and absolute methanol–ammonia T.S. (200:1 v/v) as the developing solvent has been devised for the separation of hyoscyamine and scopolamine. Quantitative evaluation of thin-layer chromatograms was achieved without elution from the adsorbent by using a direct photodensitometric method. It was demonstrated that a linear relationship exists between the size and density of the spot and the amount of substance it contained.

Total alkaloids were also determined by spectrophotometric methods, and the results were compared.

Powdered samples of *Hyoscyamus niger* L. were assayed for hyoscyamine and scopolamine by this method.

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