Belladonna Alkaloid Analysis by Partition Column Chromatography

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A sharp separation of total belladonna alkaloids (atropine, hyoscyamine, and scopolamine) from chlorpheniramine[1] and phenylpropanolamine[2] was effected by partition column chromatography which permitted the colorimetric determination of the total belladonna alkaloids. The column utilized a stationary phase of pH 7.0 buffer with moving phases of 1:1 cyclohexane-carbon tetrachloride for removal of the chlorpheniramine, and chloroform for the elution of the alkaloids. Phenylpropanolamine was retained on the column. The method was applied to pharmaceutical formulations containing the belladonna alkaloids in combination with chlorpheniramine maleate and phenylpropanolamine hydrochloride.

THE SOLANACEOUS alkaloids, atropine, hyoscyamine, and scopolamine, the well known parasympatholytic agents, have been the subject of intensive investigation evidenced by the body of literature that has grown about these substances. Analysis and/or assay methods have also been extensively reported. The two colorimetric methods most common to the reported assay procedures and used for the actual determination of the separated alkaloids are the Vitali-Morin reaction, in which a violet color is produced from the condensation of aromatic nitro-groups with acetone in the presence of potassium hydroxide (1–5), and the formation of an alkaloid or tertiary amine complex with sulfonic acid indicators (6–13). Conditions which affect the precision and accuracy of results are also discussed in the above references. These methods have been successfully adapted to the *in vivo* and *in vitro* determination of total alkaloid content.

More recently, methods have appeared in which paper chromatography has been employed for the resolution of the solanaceous alkaloids into their separate components, followed by quantitative determination with the above, or other appropriate methods (14–18). It should be mentioned that determinations using means other than color development have been used, *e.g.*, polarographic (19) and infrared (20), where conditions possible for their use were present.

The particular method chosen for the determination is governed in part by such factors as the accuracy and precision required; amount of constituent present coupled with the sensitivity of the assay method; the form encountered, *i.e.*,

crude extract, purified chemical, or pharmaceutical formulation; and the amount and nature of interfering substances. However, the problem of the actual determination many times does not resolve itself into a consideration of method advantage, but into a more basic consideration: achievement of a satisfactory separation from interfering substances so that a quantitative determination can be made.

This paper describes a method in which the total belladonna alkaloids, hyoscyamine sulfate, scopolamine hydrobromide, and atropine sulfate, were separated by partition column chromatography from a pharmaceutical preparation containing chlorpheniramine maleate and phenylpropanolamine hydrochloride. The total alkaloids were then determined colorimetrically with methyl orange as the complexing agent.

EXPERIMENTAL

Reagents.—Cyclohexane, practical grade, b.p. 71–81°.

Chloroform and carbon tetrachloride, reagent grade.

Celite (Hy-Flo Supercel, Johns-Manville Co.).

Buffer solution pH 5.6. Dissolve 27.22 Gm. of potassium dihydrogen phosphate, reagent grade, in 1 L. of distilled water and adjust to pH 5.6 with 0.1 N sodium hydroxide.

Methyl orange reagent. Saturate 1 L. of pH 5.6 buffer with approximately 2 Gm. of methyl orange, reagent grade, and filter through Whatman No. 1 filter paper. Extract the filtrate repeatedly with chloroform until the chloroform layer is colorless.

Buffer solution pH 7.0. Dissolve 5.42 Gm. of sodium dihydrogen phosphate, reagent monohydrate, and 16.36 Gm. of dibasic sodium phosphate, reagent heptahydrate, in 1 L. of distilled water and adjust to pH 7.0 with either 0.1 N hydrochloric acid or 0.1 N sodium hydroxide.

Standard solutions of alkaloids. Dry samples of hyoscyamine sulfate N.F., atropine sulfate U.S.P., and scopolamine hydrobromide U.S.P. overnight at 105°. Store the dried chemicals in a desiccator.

Stock standard. Weigh 0.869 Gm. of hyoscyamine sulfate, 0.175 Gm. of atropine sulfate, and

0.092 Gm. of scopolamine hydrobromide into a 100-ml. volumetric flask. Dissolve and dilute to volume with pH 5.6 buffer.

Intermediate working standard. Dilute 10 ml. of the stock standard to 500 ml. with pH 5.6 buffer.

Working standards. Dilute 12.5- and 25-ml. aliquots of the intermediate working standard to 100 ml. with pH 5.6 buffer. These standards represent 0.140 mg./5 ml. and 0.285 mg./5 ml. belladonna alkaloids.

Apparatus.—Klett-Summerson photoelectric colorimeter.

Chromatographic column. Approximately 19 mm. × 350 mm. with adapter equipped with a stopcock for controlling rate of flow (Ace Glass catalog No. 6261).

Rinco rotating evaporator.

Preparation of the Calibration Curve.—Transfer a 5.00-ml. aliquot of each of the two working standards to 125-ml. separators. To a third separator, transfer a 5.00-ml. aliquot of pH 5.6 buffer to serve as a reagent blank. Add 3.00 ml. of methyl orange reagent from a pipet to each funnel. Extract with 5–15-ml. portions of chloroform, collecting the chloroform extracts in a 100-ml. volumetric flask containing 15 ml. of 95% ethanol, through a pledget of cotton previously wet with chloroform. When 1-min. shake-outs are used, the chloroform layer should be essentially colorless by the fifth shake-out.

Read the color intensity of each of the standards on the Klett-Summerson photoelectric photometer, using a No. 42 filter and pH 5.6 buffer as the zero. Subtract the reagent blank from the readings obtained, and calculate the standard factor for each of the two standards by dividing the standard weight by the corrected reading. Take the average of the two. Each factor should not vary more than ±3% from the average factor.

The standard factor should be calculated daily or whenever new buffer is used.

Assay Procedure.—Accurately weigh a finely ground portion of the sample containing 0.5 to 0.7 mg. of total alkaloids into a 2-oz. mortar, add 1 ml. pH 7.0 buffer, and grind until a uniform slurry is obtained. Add 3 Gm. of Celite and 2 ml. of pH 7.0 buffer to the slurry. Regrind until the Celite is uniformly wet and homogeneous in texture.

In a second 2-oz. mortar, prepare an additional 3 Gm. of Celite by adding 3 ml. of pH 7.0 buffer and grinding until the physical appearance is as described above. Place a small mat of glass wool in the bottom of a glass chromatographic column. Transfer about 2.5 Gm. of the prepared Celite into the column and tamp firmly, but gently, with a suitable length of wood dowling.

Transfer all of the Celite containing the sample into the column: first, by scraping the mortar with a metal spatula after the loose bulk has been transferred, and adding the scrapings to the column; second, by adding the remaining Celite from the second mortar into the sample mortar, grinding the Celite around the bottom and sides of the mortar, and adding the grindings to the column as described in the initial step above. Retamp the column until the outside walls have a uniform porosity. Cap the column with a mat of glass wool.

Elute the column with 100 ml. of a mixture of 1:1 carbon tetrachloride and cyclohexane. After about 10 ml. of the mixture has been collected, stop the

flow of solvent and allow the column to equilibrate for 15 minutes, then continue until elution has ceased. Discard the mixture. Add 80 ml. of chloroform to the column and collect the eluant in a 100-ml. flat-bottom boiling flask with a standard taper 24/40 ground-glass neck. Vacuum evaporate the collected chloroform to dryness, using a rotating evaporator at room temperature. Pipet 10 ml. of pH 5.6 buffer into the flask and heat gently on a steam bath until solution of the residue is effected.

Transfer a 4-ml. aliquot of this solution into a 125-ml. separator and proceed as directed above under preparation of the calibration curve, beginning with the addition of the methyl orange reagent. Run a second 4-ml. aliquot as the sample blank using 3.00 ml. of pH 5.6 buffer in place of the methyl orange reagent. Run a reagent blank using 4.00 ml. of pH 5.6 buffer in place of the sample. Calculate the total alkaloids by

$$\frac{[\text{Klett reading} - (\text{sample blank} + \text{reagent blank})] \times \text{standard factor} \times 100}{\text{sample weight (Gm.)} \times 4} =$$

mg. total belladonna alkaloids/Gm.

Modified Vitali Method.—The use of methyl orange for the colorimetric determination was adequate since the concentration of alkaloids in the samples was relatively large. The sensitivity of the methyl orange method can be improved by reconcentrating the extracted indicator into acid (21), but because the Vitali-Morin reaction has a greater sensitivity, its use would be indicated in samples containing a concentration of alkaloids below the sensitivity of normal indicator methods. Calibration curves on the mixed alkaloids were run by the modified Vitali method (2), and Beer's law adherence was obtained down to 0.023 mg. (Fig. 1).
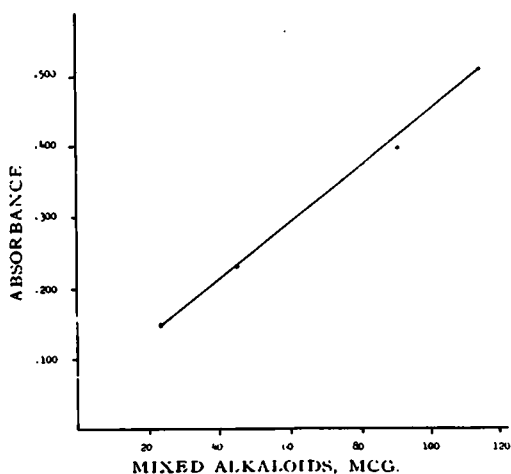

Fig. 1.—Modified Vitali method calibration curve of mixed alkaloids.

RESULTS AND DISCUSSION

Solvent partition experiments were run on hyoscyamine sulfate, chlorpheniramine maleate, and phenylpropanolamine hydrochloride at various pH conditions by equilibrating a known amount of chemical between equal volumes of the two phases. The amount remaining in the buffer phase was then determined by ultraviolet absorption. These experiments indicated that the conditions optimum to the separation of chlorpheniramine from hyoscyamine were extraction with cyclohexane at a pH of 7.0 (Table I). With the use of pH 7.6 buffer, 87% of the chlorpheniramine was extracted into the cyclohexane, but a loss of 3% hyoscyamine was encountered. Below pH 7.0, the extractability of both substances dropped markedly.

A suitable solvent extraction method was developed in which the chlorpheniramine was first removed by multiple extractions with cyclohexane from pH 7.0 buffer, followed by extraction of the alkaloids with chloroform. However, the results were uniformly high due to traces of chlorpheniramine remaining in the buffer phase, and increasing the number and volume of cyclohexane extractions served to reduce, but not eliminate, the positive error.

The distribution coefficient K for chlorpheniramine was calculated from

$$K = \frac{w_1/v}{(w - w_1)l} \qquad \text{(Eq. 1)}$$

where v is the volume in ml. of pH 7.0 buffer (phase I), w is the weight in Gm. of dissolved substance extracted with l ml. of cyclohexane (phase II), and w_1 is the solute remaining in phase I after the extrac-

TABLE I.—PARTITION EXPERIMENTS WITH pH 7.0 BUFFER

| | Per Cent in Buffer Phase | | |
	Hyoscyamine Sulfate	Chlorpheniramine Maleate	Phenylpropanolamine Hydrochloride
Chloroform	58	...	100
Carbon tetrachloride	95	10	100
Petroleum ether	100	34	100
Cyclohexane	100	31	100

tion (22). The chlorpheniramine remaining in the buffer after any given number of extractions is then

$$W_n = w \left(\frac{Kv}{Kv + l} \right)^n \qquad \text{(Eq. 2)}$$

Since the reaction of methyl orange is a molecular ratio of 1:1 in compounds having one basic amine function (23), the estimated error due to chlorpheniramine in the methyl orange reaction can be expressed as

$$E = \frac{w_n}{w_a} \times \frac{MW_1}{MW_2} \qquad \text{(Eq. 3)}$$

where w_a is the weight of alkaloids originally present with an average molecular weight of MW_1, and MW_2 is the molecular weight of chlorpheniramine maleate. From the data (Table I), K for chlorpheniramine maleate is 0.449, and calculating the estimated error after six extractions yields 10.5%. Beyond this number of extractions, simple solvent separation

TABLE II.—PARTITION COLUMN RECOVERY OF MIXED ALKALOIDS

| Determination No. | Substances Added, mg. | | | | |
	Chlorpheniramine Maleate	Phenylpropanolamine Hydrochloride	Mixed Alkaloids	Recovery, mg.	Recovered, %
1	10.0	125.0	0.599	0.587	98.0
2	10.0	125.0	0.599	0.587	98.0
3	10.0	125.0	0.599	0.602	100.5
4	10.0	125.0	0.599	0.619	103.3

TABLE III.—DATA FROM PHARMACEUTICAL FORMULATIONS

| Experiment | Theoretical Content | Initial Assay | Temperature Condition, °C. | Assay after Storage Time, mo. | | | | | | |
				1	2	3	4	6	9	12
A	0.400 mg./capsule	...	RT	...	...	...	0.395	...	...	0.403
B	0.250 mg./capsule	...	RT	0.256	0.240	0.237	0.231	0.240	0.242	0.238
		...	37°	0.259	0.244	...	...	...	...	...
		...	5°	...	0.244	...	...	...	...	...
C	0.250 mg./capsule	...	RT	0.264	0.249	...	...	...	...	...
		...	37°	0.253	...	...	...	...	...	...
		...	5°	0.251	...	...	...	...	...	...
D	0.630 mg./Gm.	0.610	...	...	...	...	...	...	...	...
E	0.250 mg./capsule	0.249	RT	0.245	...	...	...	...	...	...
F	0.250 mg./capsule	0.259	...	...	...	...	...	...	...	...
G	0.250 mg./capsule	0.251	...	...	...	...	...	...	...	...
H	0.610 mg./Gm.	0.570	...	...	...	...	...	...	...	...

was not only impractical, but showed a significant loss of hyoscyamine on known solutions due to the systemic errors inherent in the type of separation.

In order to extract the buffer exhaustively and effect the complete removal of chlorpheniramine, the technique of partition column chromatography was utilized for the separation. Recovery of the mixed alkaloids was quantitative in the presence of chlorpheniramine maleate and phenylpropanolamine hydrochloride present in the same ratio theoretically found in the samples (Table II). The mixed alkaloids in this study are susceptible to hydrolysis to form tropine, epoxy tropine, and tropic acid. Tropine and epoxy tropine react with methyl orange in the same fashion as the intact alkaloids; tropic acid does not react nor interfere with the determination.

The hydrolytic products were tested through the procedure with the result that the methyl orange blank and the hydrolysis product readings were the same. It was concluded that the degradation products were retained on the column and the method could be used as a stability procedure. The method was applied to samples of various pharmaceutical formulas for initial assay and stability determinations. The results from these experiments are listed in Table III.

Although cyclohexane was initially selected for the removal of chlorpheniramine, the mixture of cyclohexane and carbon tetrachloride was developed as the eluting solvent for two reasons: (*a*) cyclohexane caused a slower elution than desired, and (*b*) it did not remove many of the wax excipients normally associated with pharmaceutical formulations. The subsequent elution with chloroform extracted these excipients along with the alkaloids and caused hard waxy residues after the chloroform evaporation which tended to occlude the alkaloids in the pH 5.6 buffer solution.

The solvent mixture overcame the difficulties experienced with cyclohexane. The use of carbon tetrachloride itself as the eluting agent would be ideal from the standpoint of chlorpheniramine and wax removal, but the alkaloids also partition. Assays in which carbon tetrachloride was used gave low results, and decreasing the volume in an attempt to prevent the loss gave high and erratic results. The mixture selected, along with the two-stage feature of the column, accomplished a sharp separation with little interfering residue from the chloroform step.

It was found that the precision of the results was improved by equilibrating the column before each elution. The equilibration consisted of stopping the flow of eluant after 5–10 ml. had been collected and allowing the column to stand 15–20 minutes before elution was recommenced. This procedure was followed for both elutions. Time limitation necessitated the stopping of many assays during the elution with cyclohexane-carbon tetrachloride and the columns were allowed to stand overnight without affecting the results. Another advantage observed with equilibration and associated with the first elution was the further diminution of waxy residues in the chloroform eluate. Samples which

were not finely ground before being incorporated in the column showed inconsistent results even with prolonged equilibration times, and indicated that equilibration was not a substitute for a properly prepared sample.

SUMMARY

A column partition separation of the total belladonna alkaloids, hyoscyamine sulfate, atropine sulfate, and scopolamine hydrobromide, from chlorpheniramine maleate and phenylpropanolamine hydrochloride is described in which the stationary phase is pH 7.0 buffer and the moving phases are 1:1 cyclohexanecarbon tetrachloride, for the removal of chlorpheniramine, and chloroform for the elution of the alkaloids. Phenylpropanolamine does not elute with either solvent system.

The total alkaloids were determined colorimetrically using methyl orange as the complexing agent. A modified Vitali procedure is recommended for determinations in which the alkaloid content is below the sensitivity of indicator methods.

Factors governing the selection of column partition chromatography as the method of separation, choice of the eluting solvents, advantage of equilibration, and necessity for proper sample preparation are also discussed.

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