Belladonna Alkaloids and Phenobarbital Combination Pharmaceuticals Analysis I: High-Performance Liquid Chromatographic Determinations of Hyoscyamine–Atropine and Scopolamine

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Abstract □ High-performance liquid chromatographic separations are described for the analysis of hyoscyamine–atropine and scopolamine in combination pharmaceutical dosage forms containing phenobarbital. A mobile phase containing 0.034 *M* tetramethylammonium phosphate in water–methanol (21:10, pH 2.0) separated hyoscyamine or atropine from scopolamine on an octadecylsilane column in <9 min. Monitoring of the column effluent at 220 nm gave a detection limit of 0.02 μg for each alkaloid. Hyoscyamine sulfate and/or atropine sulfate were determined as total equivalent hyoscyamine sulfate, and scopolamine hydrobromide was determined as a separate entity. Data from the application of the method to commercial pharmaceutical products are also presented.

Keyphrases □ High-performance liquid chromatography—belladonna alkaloids and phenobarbital combination pharmaceuticals, determination of hyoscyamine–atropine and scopolamine □ Combination drugs—belladonna alkaloids, high-performance liquid chromatographic determination of hyoscyamine–atropine and scopolamine □ Alkaloids—belladonna, combination pharmaceuticals, high-performance liquid chromatographic determination of hyoscyamine–atropine and scopolamine

Microgram quantities of the belladonna alkaloids, hyoscyamine, atropine, and scopolamine, are widely used in combination with phenobarbital in pharmaceutical dosage forms for antispasmodic, anticholinergic, and sedative effects, especially in the treatment of functional GI disorders. Methodology was developed in this laboratory which would allow for the analysis of the belladonna alkaloids and for phenobarbital in such products. The present report presents the method, procedures, and data obtained on the determination of hyoscyamine–atropine and scopolamine.

The detection and quantitation of individual tablet levels of belladonna alkaloids in the common combination dosage forms is difficult for the following reasons: (*a*) low concentrations, (*b*) low UV absorptivity, (*c*) instability to the heat and pH ranges normally encountered in suitable sample analysis extraction procedures, and (*d*) interference from the large concentration and high UV absorptivity of phenobarbital. Currently available methodology for detection and quantitation of belladonna alkaloids includes colorimetry (1), fluorometry (2), GC (3–5), and HPLC (6, 7). The colorimetric procedure uses an ion-pair extraction with bromothymol blue at pH 5.6 for a total alkaloid determination and an ion-pair extraction with bromocresol purple at pH 6.6 for a hyoscyamine–atropine determination; scopolamine is estimated by the difference between the two assays (1). The fluorometric procedure consists of an extraction followed by ion-pair reaction with eosine yellow and determines only hyoscyamine and atropine (2). The simultaneous analysis of scopolamine and hyoscyamine–atropine can be achieved with GC but requires extensive column preparation and conditioning (3–5). Adsorption effects hamper especially the quantitation of scopolamine due to its low concentration in dosage forms as well as the peak broadening, which occurs due to its longer GC retention time. Loss of the alkaloids by adsorption on the sodium sulfate used to dry solvent extracts prior to GLC injection has also been noted (5). Due to the instability of the alkaloids' free bases, sample solutions must be injected on the day of extraction. The two HPLC (6, 7) procedures utilize reversed-phase ion-pairing chromatography with UV detection at 254 and 230 nm, respectively, to quantitate atropine and/or scopolamine. However, because of excessive background absorbance of the mobile phase, these methods lack sufficient sensitivity to quantitate the alkaloids at the microgram levels present in the common combination dosage forms.

Improved HPLC procedures are reported for the analysis of hyoscyamine, atropine, and scopolamine in combination pharmaceutical dosage forms containing phenobarbital. The method is applicable to a wide range of elixirs, tablets, and capsules for individual tablet assays (content uniformity test), as well as composite assays. In addition, the analysis of 22 commercial products using the described HPLC methods is presented.

EXPERIMENTAL

Apparatus—A liquid chromatograph[1] equipped with a 20-μl loop injector[2], a variable wavelength UV detector[3], and a recorder-integrator[4] were used. A 25 cm × 4-mm stainless steel column containing octadecylsilane chemically bonded to 5 μm silica[5] was employed.

Reagents—Methylene chloride[6] was passed through a 10 × 3-cm glass column containing 75 g of basic aluminum oxide[7] and stored over basic aluminum oxide (25 g/4 liters of solvent) in a light resistant glass container.

A carbonate buffer (pH 9.4) was prepared by mixing 5.3 g of anhydrous sodium carbonate and 4.2 g of sodium bicarbonate in 100 ml of distilled water.

A solution containing 1% concentrated HCl in methanol was prepared fresh daily.

A 0.05 *M* tetramethylammonium phosphate buffer was prepared by mixing 23 ml of 20% tetramethylammonium hydroxide in methanol[8] and 10 ml of concentrated phosphoric acid in 500 ml of distilled water, adjusting to pH 2.0 with concentrated phosphoric acid, and diluting to 1 liter with distilled water.

Preparation of Internal Standard Solutions—An internal standard solution was prepared for use in the drug standard solution by dissolving anhydrous theophylline[9] in methanol (300 μg/ml). The theophylline

[1] Constametric I Pump, Laboratory Data Control, Riviera Beach, Fla.
[2] Chromatography Accessory Module Injector, 20-μl loop, Laboratory Data Control, Riviera Beach, Fla.
[3] Spectromonitor II Variable Wavelength UV-Visible Detector, Laboratory Data Control, Riviera Beach, Fla.
[4] HP 3380A Integrator, Hewlett-Packard, Palo Alto, Calif.
[5] Spherisorb octadecylsilane, 5 μm. Prepacked column purchased from Laboratory Data Control, Riviera Beach, Fla.
[6] Burdick and Jackson Laboratories, Muskegon, Mich.
[7] Brockmann Activity Grade 1, J. T. Baker, Phillipsburg, N.J.
[8] Aldrich Chemical Co., Milwaukee, Wis.
[9] K&K Laboratories, Plainview, N.Y.

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Table I—Recovery Data for Spiked Sample Determinations of Commercial Formulations [a]

Elixirs			Tablets and Capsules		
	Recovery, %			Recovery, %	
Formulation	Hyoscyamine Sulfate	Scopolamine Hydrobromide	Formulation	Hyoscyamine Sulfate	Scopolamine Hydrobromide
1	97.2	93.7	11	99.1	—[b]
2	101.1	94.3	12	94.8	99.6
3	101.0	98.0	13	102.7	94.3
4	100.6	106.1	14	101.0, 99.3[c]	95.7, 97.4[c]
5	102.7	93.0	15	94.4	93.6
6	105.1	[b]	16	98.3	106.1
7	94.7	[b]	17	99.0	104.4
8	95.6	96.5	18	102.2	105.2
9	97.3	93.3	19	99.8	100.4
10	103.1	[b]	20	99.3	95.6
—			21	98.2, 98.3[c]	102.8, 101.2[c]
—			22	103.0	—[b]
Mean Recovery	99.8	96.4		99.3	99.8
SD	3.46	4.65		2.75	4.74
RSD	±3.47	±4.82		±2.77	±4.75
Range	94.7–105.1	93.0–106.1		94.4–103.0	93.6–105.2

[a] Portions of sample spiked with standard solution containing hyoscyamine sulfate and scopolamine hydrobromide and analyzed according to procedure. [b] Product did not contain scopolamine hydrobromide. [c] Value obtained by second analyst. Value not included in statistics.

solution was then diluted with methylene chloride (2.25 μg/ml) for use in the assay procedure.

Preparation of Drug Standard Solutions—A standard solution of scopolamine hydrobromide[10] in distilled water (500 μg/ml) was prepared and stored in low-actinic glassware.

A mixed standard solution was prepared by weighing hyoscyamine sulfate[11] (16 mg) into a 50-ml low-actinic volumetric flask, adding 3.0 ml of the methanolic theophylline internal standard solution (300 μg/ml) and 2.0 ml of scopolamine hydrobromide standard solution, and diluting to ~40 ml with distilled water.

Preparation of Mobile Phase—The mobile phase was prepared by mixing 525 ml of 0.05 *M* tetramethylammonium phosphate buffer with 250 ml of methanol.

HPLC Conditions—A flow rate of 0.8 ml/min was used with UV detection set at 220 nm and the sensitivity set at 0.02 aufs.

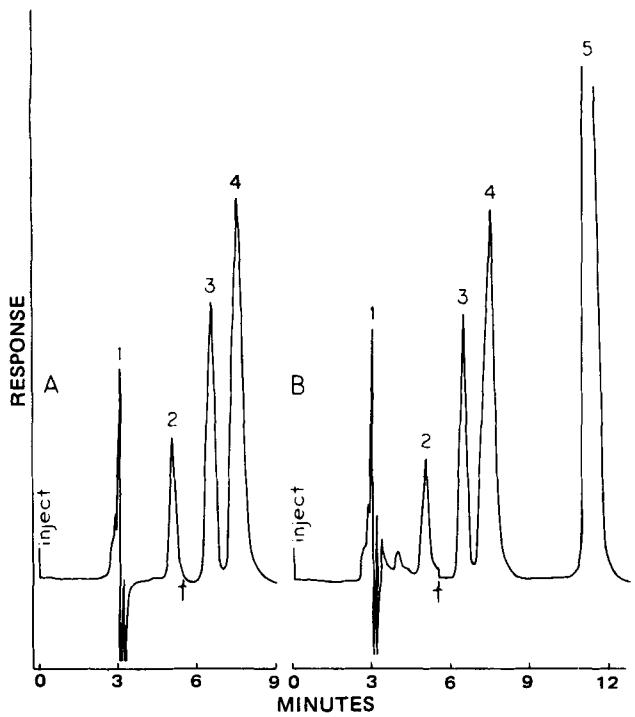

Figure 1—*Typical chromatograms of HPLC separations of A, the standard solution and B, the tablet sample. Key: 1, solvent; 2, scopolamine; 3, theophylline (internal standard); 4, hyoscyamine–atropine; 5, phenobarbital. Attenuation change of 4× after each scopolamine peak at the arrow.*

[10] USP Reference Standard.
[11] NF Reference Standard.

Assay for Tablets and Capsules—Twenty tablets were weighed and finely powdered, or the contents of 20 capsules were weighed and composited. A sample portion equivalent to ~0.6 mg of combined hyoscyamine sulfate–atropine sulfate was accurately weighed and quantitatively transferred to a 50-ml centrifuge tube; 25 ml of 0.05 *N* sulfuric acid then was added and the tube shaken for 15 min. The mixture was then centrifuged for 5 min at 3000 rpm. A 5.0-ml aliquot of the supernate was pipeted into a 125-ml separatory funnel where it was extracted with two 30-ml portions of methylene chloride. The organic phase was then discarded. Two milliliters of carbonate buffer (pH 9.4) was added to the aqueous phase remaining in the separatory funnel and the mixture was extracted with four 30-ml portions of methylene chloride. Each extract was filtered through a methylene chloride-rinsed glass wool plug into a flash evaporator[12] fitted with a concentrator tube[13]. Exactly 3 ml of theophylline internal standard solution (2.25 μg/ml) and 9–10/50 grit boiling chips[14] were added to the concentrator. A distilling column[15] was added to the concentrator and the methylene chloride evaporated on a steam bath to ~10 ml. The distilling column was then rinsed with 1–2 ml of methylene chloride and the solvent evaporated to 0.5–1 ml. The distilling column was removed and the column-concentrator junction rinsed with ~1 ml of methylene chloride into the concentrator tube. The concentrator tube was placed in a beaker of warm water (40 ± 5°) and the solvent evaporated to ~1 ml with the aid of a current of dry air. Next, 0.1 ml of acidic methanol was added, mixed, and the solution evaporated to dryness using the conditions described previously. The sides of the concentrator tube were rinsed with 0.5 ml of methanol and the solution again evaporated to dryness. The extract was then dissolved in 300 μl of distilled water and a 20-μl sample was injected onto a liquid chromatograph.

Content Uniformity Test—One tablet or the contents of one capsule were transferred to a 125-ml separatory funnel and 7 ml of 0.05 *N* sulfuric acid added. The funnel was shaken until the tablet disintegrated and then shaken for an additional 5 min. Thereafter, the "Assay for Tablets and Capsules" was followed beginning with, " . . . it was extracted with two 30-ml portions of methylene chloride . . .."

Assay for Elixirs—A sample equivalent to ~0.6 mg of combined hyoscyamine sulfate–atropine sulfate was pipeted into a 150-ml beaker. The solution was warmed in a 40 ± 5° water bath with a current of air for 30 min to remove alcohol. After cooling to room temperature, the sample was quantitatively transferred to a 25-ml volumetric flask and adjusted to volume with distilled water. Exactly 5 ml of this solution was pipeted into a 125-ml separatory funnel, 2 ml of 0.2 *N* sulfuric acid added, and the "Assay for Tablets and Capsules" was followed thereafter beginning with, " . . . it was extracted with two 30-ml portions of methylene chloride . . .."

Calculations—After obtaining the chromatograms, each peak response was determined by measurement of peak height or peak area. The quantity, in milligrams, of hyoscyamine sulfate–atropine sulfate and scopolamine hydrobromide in the portion of sample taken was calculated

[12] Kuderna-Danish Concentrator, Kontes Glass Co., Vineland, N.J.
[13] Mills Tube, Kontes Glass Co., Vineland, N.J.
[14] Carborundum.
[15] Snyder Distilling Column, Kontes Glass Co., Vineland, N.J.

Table II—HPLC Analysis Results for Commercial Formulations

Formulation	Form	Percent of Label Claim: Hyoscyamine Sulfate[a]–Atropine Sulfate	Percent of Label Claim: Scopolamine Hydrobromide[b]
1	Elixir	89.3	93.2
2	Elixir	106.6	88.6
3	Elixir	90.2	105.5
4	Elixir	101.1	100.7
5	Elixir	93.2	81.7
6	Elixir	105.1	[c]
7	Elixir	91.8	[c]
8	Elixir	95.3	81.4
9	Elixir	32.1	88.2
10	Elixir	106.4	[c]
11	Tablet	93.6, (95.2)[d]	[c]
12	Tablet	93.2, (94.7)	97.5, (95.2)[d]
13	Tablet	91.9, (92.5)	101.2, (100.6)
14	Tablet	99.4, 93.1[e], (98.1)	100.0, 94.5[e], (90.5)
15	Tablet	83.7, (85.2)	[c]
16	Tablet	95.8, (96.2)	83.2, (87.4)
17	Tablet	101.1, (97.7)	99.2, (99.4)
18	Tablet	95.5, (91.7)	103.4, (97.1)
19	Tablet	90.5, (89.3)	55.5, (50.5)
20	Tablet	91.6, (94.8)	88.9, (98.0)
21	Capsule	98.2, 96.2[e], (100.9)	90.0, 98.3[e], (104.6)
22	Capsule	11.7, (10.8)	[c]

[a] Label claim range from 0.1205–0.217 mg/unit dose. [b] Label claim range from 0.0060–0.0284 mg/unit dose; 0.006–0.0075 most common. [c] Product did not contain scopolamine hydrobromide. [d] Values in parentheses are the average of 10 individual tablet assays (content uniformity). [e] Value obtained by second analyst.

by the formula $0.3C \times (Ru/Rs)$. The values Ru and Rs are the ratios of the hyoscyamine sulfate (or scopolamine hydrobromide) response to the internal standard response for sample (u) and standard (s), respectively, and C is the concentration of hyoscyamine sulfate (or scopolamine hydrobromide) (milligrams per milliliter) in the standard solution.

RESULTS AND DISCUSSION

Mobile Phase Selection—The presence of tetramethylammonium ions at pH 2 in the methanol–water mobile phase was found to minimize column adsorption of the alkaloids resulting in decreased retention time. Their presence also provided a high resolution separation of scopolamine and hyoscyamine–atropine, as shown in Fig. 1. The mobile phase was sufficiently UV-transparent for the detector to be set at 220 nm where alkaloid absorptivity is considerably higher. Three octadecylsilane columns were used, and the separation of component peaks on each column was as good as or better than that shown.

Even though the pH of the mobile phase buffer was at the lower extreme of the range normally considered advisable for column stability, the columns maintained separation and reproducibility for 5 months with daily use. At the end of each day's use, the column was rinsed with water and then methanol and stored in methanol overnight to avoid column deterioration from prolonged contact with the low pH buffer in the mobile phase.

Interferences—Considerable interference in the area of the hyoscyamine–atropine peak was encountered when first testing the procedure. Upon checking the reagent blank, the interference was attributed to impurities in commercially available methylene chloride. Passing the methylene chloride through basic alumina eliminated the interference. Each commercial product analyzed was carried through the procedure without addition of internal standard to check for interference of excipients in the area of the internal standard peak. A synthetic preparation of excipients representative of those encountered in the commercial products analyzed was also prepared and carried through the procedure to test for interference in the areas of the scopolamine peak and hyoscyamine–atropine peak. No significant interferences were detected. Phenobarbital was not entirely removed by the extraction procedure but did not interfere with the HPLC determination because it was well separated from the peaks of interest, as shown in Fig. 1.

Stability of Standard Preparation and Sample Solutions—A standard solution was prepared and its peak areas checked each day against a freshly prepared standard solution for 1 week. No deterioration in standard peak responses was noted. A sample solution was treated in a similar manner and sample peak responses also did not deteriorate. When standards or samples were dissolved in 0.05 N sulfuric acid or mobile phase instead of water, peak responses deteriorated.

Validation Tests—Twenty microliters of each of a series of five standard solutions containing 0–0.5 mg of hyoscyamine sulfate/ml and 0–0.032 mg of scopolamine hydrobromide/ml were injected onto the HPLC. Linear responses were obtained for each drug in its respective range by both peak height and peak area. Six replicate injections of the procedural standard solution gave a coefficient of variation of 1.4% for scopolamine hydrobromide and 0.5% for hyoscyamine sulfate.

Precautions in Sample Extraction—To minimize decomposition of the alkaloids due to heat, acids, and bases, the sample was carried through the entire extraction procedure to final dilution without stopping. The evaporation of solvent was found to be the most critical step. Since the alkaloid free bases decompose above 45°, methylene chloride (bp 38°) was chosen as the extracting solvent. Use of the flash evaporator allowed for rapid evaporation without bumping and splattering of the solvent. During the evaporation, the sample was not allowed to go to dryness before addition of methanolic hydrochloric acid to prevent possible loss of the alkaloids' free bases. The final methanol rinse was added to drive off residual hydrochloric acid which would decompose scopolamine salt. Because the alkaloid concentrations are in the microgram range, and thus subject to minute interferences, all glassware was thoroughly rinsed with distilled water and methylene chloride to ensure cleanliness.

Recovery of Standards—An aliquot of each commercial formulation assay composite was spiked with a standard solution of scopolamine hydrobromide and hyoscyamine sulfate, extracted and assayed according to the procedure, and the percentage of standard recovered calculated. Recovery of alkaloid standards was also checked by a second analyst for Formulations 14 and 21. The recovery results for each product and the statistical evaluation of the recovery data are summarized in Table 1.

Analysis of Commercial Formulations—Twenty-two commercial formulations consisting of elixirs, tablets, and capsules were assayed. Tablets and capsules were also analyzed for content uniformity. Results of the analyses are summarized in Table II. The average content uniformity values generally agreed with the assay values, but individual tablet values often varied ±10% of the average value. This variation coupled with the low concentration of alkaloids caused difficulty in preparing a uniform assay composite for tablets and capsules. Assays on Formulations 14 and 21 were checked by a second analyst, and the results compared favorably with those of the first analyst.

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