ipodate by the U.S.P. XVI heavy metals test, method II. The heavy metals limit for calcium ipodate is 30 p.p.m.

Assay.—Transfer about 300 mg. of calcium ipodate, accurately weighed, to a glass-stoppered, 250-ml. flask, add 30 ml. of sodium hydroxide solution (1 in 20) and 500 mg. of powdered zinc, connect the flask to a reflux condenser and reflux the mixture for 30 min. Cool to room temperature, wash the condenser with 20 ml. of water, and filter the mixture. Wash the flask and the filter with small portions of water, adding the washings to the filtrate. Add to the filtrate 5 ml. of glacial acetic acid and 3 drops of eosin Y T.S., and titrate with 0.05 *N* silver nitrate until the entire mixture changes to a permanent pink color. Each milliliter of 0.05 *N* silver nitrate is equivalent to 6.345 mg. of iodine (I), and to 10.28 mg. of $C_{24}H_{24}CaI_6N_4O_4$. The amount of calcium ipodate found, calculated on the anhydrous basis, is not less than 97.5% and not more than 102.5% of the weight of the sample taken.

DOSAGE FORMS OF CALCIUM IPODATE

Calcium Ipodate Granules

Identity Tests.—Transfer to a beaker an amount of calcium ipodate granules equivalent to about 2 Gm. of calcium ipodate, add 100 ml. of water and stir for several minutes. Filter the suspension with the aid of suction and wash the filter with several small portions of water. Dry the residue of calcium ipodate in a vacuum oven at 60° for 4 hr. The residue responds to the identity tests in the monograph for calcium ipodate.

Assay.—Accurately weigh the contents of not less than 10 packets of calcium ipodate granules, and mix well. Transfer an accurately weighed portion of the granules, equivalent to about 300 mg. of calcium ipodate, to a glass-stoppered, 250-ml. conical flask and proceed as directed in the assay under calcium ipodate beginning with "... add 30 ml. of sodium hydroxide solution (1 in 20) ..." Each milliliter of 0.05 *N* silver nitrate is equivalent to 10.28 mg. of $C_{24}H_{24}CaI_6N_4O_4$. The amount of calcium ipodate found is not less than 85.0% and not more than 115.0% of the labeled amount.

DISCUSSION

Calcium ipodate[1] is an oral radiographic contrast medium for cholangiography and cholecystography.

A brief discussion of the identity tests and quantitative methods may be found in the monograph for sodium ipodate (1).

The assay of bulk calcium ipodate gave an average value of 99.7 ± 0.1%,[2] equivalent to 61.5 ± 0.1%[2] iodine (I). Analysis of commercial 3-Gm. packets gave an average value of 87.9 ± 1.9%[2] of the labeled amount of calcium ipodate.

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(1) *J. Pharm. Sci.*, **54**, 909(1965).

[1] Marketed as Oragrafin Calcium by E. R. Squibb and Sons New York, N. Y.

[2] Maximum deviation from the mean value.

Chromatographic Analysis of Phenobarbital and Belladonna Alkaloid Combinations

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A partition column chromatographic technique is applied to the analysis of phenobarbital and the belladonna alkaloids as found in commercial tablets and elixirs. Separation is achieved on a Celite column in which the stationary phase is a 10 per cent solution of *p*-toluenesulfonic acid. Ether and chloroform elute phenobarbital and the alkaloid–*p*-toluenesulfonic acid complex, respectively. The isolated phenobarbital is determined spectrophotometrically, and the total belladonna alkaloids are measured colorimetrically by a modification of a published procedure. A study of the modifications is reported and statistically evaluated. Standard recoveries averaged 99.01 per cent for phenobarbital and 98.73 per cent for atropine sulfate.

THE BELLADONNA alkaloids are commonly formulated in combination with phenobarbital.[1] Current analytical procedures for the assay of such a specific formulation appear to be limited to conventional extraction-titration methods (1), although many techniques have been reported for the assay of a variety of mixtures containing only one of the components.

Methods for the determination of atropine in combinations include several titration or colorimetric determinations (2), some of which are utilized in the official compendia. Basu and Dutta (3) separated morphine from atropine with reineckate salts at controlled pH values. Ion-exchange resins have been successfully utilized by several investigators in the isolation

Received January 28, 1965, from the Division of Pharmaceutical Chemistry, Bureau of Scientific Research, Food and Drug Administration, U. S. Department of Health, Education, and Welfare, Washington, D. C.

Accepted for publication April 26, 1965.

Based on a thesis submitted by Stanley A. Koch to the Graduate School, University of Maryland, Baltimore, in partial fulfillment of Master of Science degree requirements.

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[1] This paper is concerned with those preparations compounded with the salts of the individual alkaloids, atropine, hyoscyamine, and hyoscine. Products containing fluidextract or tincture of belladonna were not included in these studies.

and in the purification of the tropane alkaloids from multicomponent mixtures (4, 5), as have the adsorbents magnesium oxide (6), silica gel (7), and alumina (8). Sterescu and Popovici (9) reported the separation and determination of atropine and scopolamine in mixtures with papaverine and sparteine by partition paper chromatography. However, the most promising methods of quantitative separation of many such complex mixtures have been achieved with column partition chromatography. Using this technique, Levine and Roe (10) separated atropine from tropic acid, and Santoro and Matz (11) separated the belladonna alkaloids from chlorpheniramine maleate and phenylpropanolamine hydrochloride. The usefulness and potential of partition chromatography have likewise been illustrated in mixtures containing phenobarbital (12). Heuermann and Levine (13) separated mixtures containing acetylsalicylic acid, acetophenetidin, caffeine, and phenobarbital by using two columns in series.

DISCUSSION

The column chromatographic method described here permits the separation, isolation, and direct quantitative determination of both phenobarbital and the belladonna alkaloids. A solution of the sample in 10% aqueous *p*-toluenesulfonic acid constitutes the immobile phase. The sample column is eluted with ether, which removes the neutral and acidic extractives, including phenobarbital, without disturbing the alkaloids. The effluent is passed onto a Celite:2/3 *M* K_3PO_4 column, which effectively retains phenobarbital as its potassium salt. The more acidic components (including coloring agents), which would also be retained on the tribasic potassium phosphate column and interfere with the subsequent measurement of the phenobarbital, are trapped on an intermediate column containing a 25% dibasic potassium phosphate immobile phase, which is not sufficiently basic to trap the phenobarbital. Neutral extractives pass through both columns and are discarded.

The basicity of the K_3PO_4 column is reversed *in situ* with acetic acid in chloroform, liberating the phenobarbital from its salt and effecting its elution in the chloroform. The phenobarbital must be eluted without delay, since hydrolysis occurs in the alkaline medium within 30 min. The phenobarbital in the effluent is measured spectrophotometrically in an aqueous solution at about pH 10, where it shows a characteristic ultraviolet absorption peak at 240 mμ.

The belladonna alkaloid–*p*-toluenesulfonic acid complex is eluted from the sample column with chloroform, as described by Levine and Ottes (14). The eluate is passed onto a separate Celite column containing sodium hydroxide solution as the stationary phase; this retains the *p*-toluenesulfonic acid moiety, and allows the isolated alkaloid to be collected in the eluate.

The method of analysis adopted for the determination of the isolated belladonna alkaloids is the colorimetric procedure reported by Levine and Roe (10). The color development is dependent upon the aromatic ring of the tropic acid moiety which is quantitatively nitrated with fuming nitric acid. The resulting nitro compound is reduced with sodium hydrosulfite to the corresponding amine, which is diazotized and coupled with Bratton-Marshall reagent to form the characteristic color. Hydrosulfite destroys this color; nitrous acid and nitrogen oxides intensify it (usually producing a fugitive color). Complete removal of these is, therefore, essential.

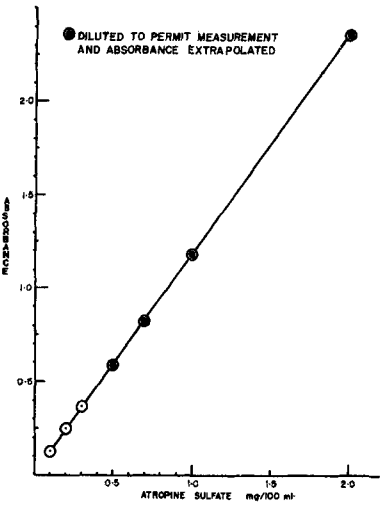

Fig. 1.—Calibration curve of atropine sulfate using the modified Levine-Roe colorimetric procedure.

In applying the colorimetric procedure to standard samples of atropine sulfate, it was noted that the absorptivities of the final colored solution varied significantly from day to day. The procedure was, therefore, critically investigated to find and eliminate the cause of these variations. Although no single operation was found to be entirely responsible for them, a significant improvement in the reproducibility was obtained by making three modifications of the original procedure: (*a*) the timed reduction of the nitro group is conducted at room temperature, avoiding heat which may accelerate auto-oxidation of the hydrosulfite and increase the possibility of reductive side reactions; (*b*) diazotization is performed for a controlled period at close to 0° to minimize side reactions (*e.g.*, decomposition of the diazo compound to the corresponding phenol and coupling of the latter with the diazotized amine); and (*c*) the quantity of coupling reagent added to each sample is controlled.

Because of the sensitivity of the color reaction, scrupulously clean glassware must be used, and correction must be made for background color resulting from solvent impurities and from interfering substances extracted from the Celite. To make this correction, a blank determination, using the entire chromatographic procedure, is run in parallel with the sample determination.

It was found that each of these modifications resulted in an improvement of the color reproducibility to a degree which is statistically significant. The colorimetric procedure as originally described gave results with a standard deviation of 13.70 ×

TABLE I.—DETERMINATION OF PHENOBARBITAL AND ATROPINE SULFATE IN KNOWN MIXTURES

Sample	Substance	Amt. Added, mg.	Amt. Recovered, mg.	Recovery, %
1	Phenobarbital	30.81	30.6	99.3
	Atropine sulfate	0.3000	0.294	98.0
2	Phenobarbital	30.81	30.6	99.3
	Atropine sulfate	0.3000	0.297	99.0
3	Phenobarbital	31.34	31.1	99.2
	Atropine sulfate	0.3000	0.298	99.3
4	Phenobarbital	31.00	30.8	99.4
	Atropine sulfate	0.3000	0.297	99.0
5	Phenobarbital	30.90	30.6	99.0
	Atropine sulfate	0.3000	0.296	98.7
6	Phenobarbital	31.84	31.3	98.3
	Atropine sulfate	0.3000	0.297	99.0

10^{-3}. By controlling the above listed parameters, the standard deviation was reduced to 1.48×10^{-3}, and the coefficient of variation was decreased from 4.07 to 0.41%. The color produced obeys the Beer-Lambert law over a wide range. (Fig. 1.)

METHOD

Chromatographic Column.—Fuse a 5-cm. length of 6- to 8-mm. glass tubing to a 25 × 250 mm. test tube. Pack a pledget of fine glass wool in the base of column as support.

Tamping Rod.—A disk of stainless steel, aluminum, or glass with a diameter slightly less than that of column, attached to a handle 12–18 in. long.

Standard Solutions

Phenobarbital.—Transfer about 10 mg. of phenobarbital U.S.P., accurately weighed, to a 100-ml. volumetric flask and dissolve in about 20 ml. of water containing 10 drops of ammonium hydroxide. Adjust to volume with water, and dilute a 10-ml. aliquot to 100 ml. Prepare this standard solution shortly before use.

Atropine.—Prepare, by stepwise dilution, a solution containing about 0.1 mg./ml. of U.S.P. atropine sulfate reference standard. Evaporate a 3-ml. aliquot to dryness in a 250-ml. conical flask.

Preparation of Columns

Column A.—*Tablets.*—Weigh into a 150-ml. beaker a quantity of powdered tablets representing about 0.3 mg. of atropine sulfate. Add 5 ml. of 10% *p*-toluenesulfonic acid solution, and stir to dissolve the soluble components. Add 5 Gm. of Celite[2], mix thoroughly, and transfer to the column. Dry-wash the beaker with about 1 Gm. of Celite and transfer it to the column. Tamp, using gentle pressure, to a uniform mass, and cover with a mat of glass wool. Prepare a separate column without sample for column blank.

Elixir.—Transfer to a 150-ml. beaker an amount of elixir equivalent to about 0.3 mg. of atropine sulfate. Heat gently on a steam bath to remove the alcohol, and reconstitute to the original volume with water. Dissolve 1 Gm. of *p*-toluenesulfonic acid in the sample solution, add 7 Gm. of Celite, mix thoroughly, transfer to a column and continue as directed under *Tablets*, beginning with "Dry-wash . . . ."

Column B.—Mix 3 Gm. of Celite with 2 ml. of 25% K_2HPO_4 (w/v) solution. Transfer to a column and tamp as above.

[2] Acid washed Celite 545, Johns-Manville Corp., used throughout.

Column C.—Six grams of Celite and 5 ml. of 2/3 *M* K_3PO_4.

Column D.—Four grams of Celite and 3 ml. of 1 *N* NaOH. Prepare a separate column for column blank.

Wash columns B, C, and D with 50 ml. of ether (water-saturated), followed by 50 ml. of water-saturated chloroform (obtained from the distillation of ACS reagent grade chloroform with about one-fourth its volume of approximately 1 *N* H_2SO_4).

Mount columns A, B, and C in series so that effluent from column A flows through column B, then onto column C.

Procedure

Separation of Components.—(Use water-saturated solvents throughout.) Pass 150 ml. of ether over columns A, B, and C, and over blank column A, and discard the eluate. Separate the columns, and place a 250-ml. flask as receiver under column C. Without delay, elute the phenobarbital with a solution of 0.5 ml. of glacial acetic acid in 10 ml. of chloroform, followed by a solution of 0.5 ml. of the acid in 100 ml. of chloroform. Evaporate the eluate to dryness on a steam bath under a current of air.

Mount column A directly over column D and elute the belladonna alkaloids with 100 ml. of chloroform, collecting the eluate in a 250-ml. conical flask. Add 2 drops of hydrochloric acid and evaporate the solution to dryness. Treat blank columns A and D in the same manner.

Determination of Phenobarbital.—Dissolve the residue obtained from column C in about 25 ml. of water containing 10 drops of ammonium hydroxide. Transfer the solution to a 250-ml. volumetric flask and dilute to volume with water. Dilute a 10.0-ml. aliquot to 100.0 ml. and measure its absorbance at the maximum at about 240 mμ. Concomitantly determine the absorbance of the standard solution.

Determination of Belladonna Alkaloids.—Add 1.0 ml. of fuming nitric acid (90%) to the flasks containing the alkaloid residue (eluate from column D), and the atropine sulfate standard, and to a blank flask. Cover each with a watch glass and heat on a vigorously boiling steam bath for 30 min. Remove from the heat and add 10 ml. of water, 2 ml. of ammonium hydroxide, and about 50 mg. of sodium hydrosulfite (conveniently measured with a calibrated microscoop). Mix to dissolve the hydrosulfite and allow to stand at room temperature for 20 min. Add 5.0 ml. of 5% sodium nitrite solution and several pieces of chopped ice

TABLE II.—DETERMINATION OF PHENOBARBITAL AND BELLADONNA ALKALOIDS IN COMMERCIAL SAMPLES[a]

Sample[b]	Substance	Labeled[c] Amt., mg./Tablet	Amt. Found, mg./Tablet	Labeled Amt., %	Av. %
1	Phenobarbital	16.0	15.8	98.8	
			15.9	99.4	
			15.9	99.4	99.2
	Belladonna alkaloids	0.1562	0.153	97.9	
			0.153	97.9	
			0.153	97.9	97.9
2	Phenobarbital	16.2	15.6	96.3	
			15.6	96.3	
			15.6	96.3	96.3
	Belladonna alkaloids	0.1256	0.142	113.1	
			0.142	113.1	
			0.142	113.1	113.1
3	Phenobarbital	16.0	15.8	98.8	
			15.7	98.1	
			15.7	98.1	98.3
	Belladonna alkaloids	0.1498	0.153	102.1	
			0.153	102.1	
			0.152	101.5	101.9
4	Phenobarbital	16.2	15.8	97.5	
			15.8	97.5	
			15.8	97.5	97.5
	Belladonna alkaloids	0.1251	0.124	98.7	
			0.124	98.7	
			0.123	97.9	98.4
5	Phenobarbital	16.0	15.9	99.4	
			15.9	99.4	
			15.8	98.8	99.2
	Belladonna alkaloids	0.1879	0.184	97.9	
			0.182	96.9	
			0.183	97.4	97.4
6	Phenobarbital	16.2	15.6	96.3	
			15.7	96.9	
			15.7	96.9	96.7
	Belladonna alkaloids	0.1256	0.122	97.1	
			0.121	96.3	
			0.121	96.3	96.6
7	Phenobarbital	16.2/5 ml.	16.2	100.0	
			16.1	99.4	
			16.2	100.0	99.8
	Belladonna alkaloids	0.1256/ 5 ml.	0.124	98.7	
			0.125	99.5	
			0.127	101.1	99.8

[a] A typical formulation of these products is as follows: atropine sulfate, 0.0194 mg.; hyoscyamine sulfate, 0.1037 mg. scopolamine hydrobromide, 0.0065 mg.; phenobarbital, 16.2 mg. [b] Tablets, *1–6*; elixir, *7*. [c] Weight of belladonna alkaloid expressed as the equivalent weight of atropine sulfate.

(prepared from deionized water). Add 1.5 ml. of concentrated hydrochloric acid serially at 2-min. intervals and let stand for exactly 30 min., adding ice as needed to maintain the temperature below 5°. Add 10 ml. of 5% sulfamic acid to the flasks in the same order as above, shake, and dispel nitrous oxide fumes from flasks with a current of air. Immediately add 4.0 ml. of a freshly prepared 0.8% solution of *N*-(1-naphthyl)-ethylenediamine dihydrochloride. Transfer to a 100-ml. volumetric flask, dilute to volume, and determine the absorbance at 550 mμ within 0.5–4 hr. Correct the absorbance of the standard for that of the reagent blank and the absorbance of the sample for that of the column blank.

RESULTS

Standard mixtures of phenobarbital and atropine sulfate approximately equivalent to those found in commercial tablet formulations were assayed. The results are presented in Table I. The assay values of commercial dosage forms containing such a phenobarbital–belladonna alkaloid formulation are reported in Table II.

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