tation since the results are also compatible by this mechanism.

Edetate disodium has promoted an increase in intestinal (rat) absorption of salicylate anion (9), phenolsulfonphthalein (10), heparin (11), mannitol (11), inulin (11), decamethonium (11), and sulfanilic acid (11). It has been postulated that edetate disodium alters the aqueous permeability of the intestinal epithelium by depleting magnesium and calcium ions, resulting in separation of the epithelial cells of the rat intestine (12, 13). This mechanism is likewise suspected with acetazolamide, since the lack of effect of edetate disodium in inhibiting the binding of acetazolaminde to human carbonic anhydrase discounts the possibility of enzyme inactivation by removal of zinc ion. The possibility of edetate disodium acting as an *in vivo* inhibitor to the binding of acetazolamide to carbonic anhydrase is made more difficult by the fact that an endogenous source of free calcium exists to bind preferentially to the divalent edetate and to prevent calcium removal from the enzyme.

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GLC Assay of Belladonna Extracts

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Abstract □ Samples of belladonna pilular and powdered extract and tincture from two suppliers were analyzed by GLC as a cooperative effort between two laboratories to develop methodology with substantial improvements in sensitivity, specificity, precision, and working time over the present official method. This goal was achieved, but marked differences in response of the individual extracts to different isolation schemes were noted.

Keyphrases □ Belladonna extract—GLC analysis in pharmaceutical formulations, suitability of anhydrous sodium sulfate in isolation, compared to official method □ Alkaloids, belladonna—GLC analysis in pharmaceutical formulations, suitability of anhydrous sodium sulfate in isolation, compared to official method □ Atropine—GLC analysis, pharmaceutical formulations of belladonna extract □ Scopolamine—GLC analysis, pharmaceutical formulations of belladonna extract □ Hyoscyamine—GLC analysis, pharmaceutical formulations of belladonna extract □ GLC—analysis, belladonna alkaloids, pharmaceutical formulations

Reliable GLC assays (1, 2) of atropine and scopolamine in dosage forms have come into general use in recent years and have been the basis of extensions (3) to other drug mixtures. Official titrimetric assays (4, 5) for the crude belladonna extracts (powdered and pilular) and the directly derived tincture are tedious, unspecific, and unreliable because of emulsion problems. Application of the GLC method to these mixtures of plant extracts would be desirable, and this paper reports the results of a cooperative effort between two laboratories to accomplish this goal. Some earlier assay work on plant extracts from belladonna by a GLC method was reported (6), and inaccuracies of the titrimetric approach also were noted then.

EXPERIMENTAL

All reagents were USP, NF, or ACS grade. Samples of pilular and powdered extracts were received from manufacturers, and the tincture was obtained locally. The optical isomers in atropine are not separated, so the combined atropine–hyoscyamine peak is expressed solely as atropine herein for convenience.

Chromatographic System[1]—A 1.2-m × 4-mm i.d. glass column packed with 3% methyl phenyl polysiloxane oil on 100–120-mesh acid-washed, flux-calcined, diatomaceous earth[2] was cured and conditioned as specified elsewhere (1, 2, 5). Minimal pledgets of silanized glass wool were used to minimize the catalytical conversion of atropine to apoatropine (6, 7). The column was maintained at 215°, and the injection port and flame-detector block temperatures were 240 and 245°, respectively; dry helium was used as a carrier gas at a flow rate of 65 ml/min. Electronic peak measurement was employed[3].

System Suitability—Chromatograph six to 10 injections of the assay preparation, and record peak areas as directed under *Procedure*. The analytical system is suitable for conducting this assay if the relative standard deviation for the ratio, R_A, calculated by the formula 100 × (standard deviation/mean ratio), does not exceed 2.0%; the resolution factor between A_H and A_A is not less than 5; and the tailing factor (the sum of the distances from peak center to the leading edge and to the tailing edge divided by twice the distance from the peak center to the leading edge), measured at 5% of the peak height of A_A, does not exceed 2.0.

[1] HP 5750B gas chromatograph with flame-ionization detector fitted for on-column injection.

[2] OV-17 on Gas Chrom Q, Applied Science Laboratories.

[3] Infotronics CRS 204 digital integrator.

Anhydrous Sodium Sulfate—Transfer about 10 mg of atropine base, accurately weighed, to a 25-ml volumetric flask. Dilute to volume with 95% ethanol. Pipet 3.0 ml of this solution into each of two 60-ml separators and add about 10 ml of water, 1 ml of 1 *N* sodium hydroxide, and 10 ml of chloroform. Shake vigorously and allow the layers to separate. Then filter the organic phase from the first separator (Solution A) through phase-separating paper[4], suitably supported in a funnel, into a container and filter that from the second separator (Solution B) through about 30 g of anhydrous sodium sulfate (previously chloroform washed), supported with a small pledget of glass wool in a funnel, into a suitable container.

Extract a second time with 10 ml of chloroform and again collect the organic phase in a similar fashion. Evaporate the combined organic phases *in vacuo* to dryness and immediately add 1.0 ml of chloroform. Inject Solution A in duplicate and record the average peak area. Similarly inject and record Solution B. For the batch of reagent to be acceptable for use in this procedure, the two assay areas must be within 5% of each other.

Internal Standard Solution—Dissolve about 40 mg of USP homatropine hydrobromide reference standard, accurately weighed, in 0.1 *N* H_2SO_4 contained in a 50-ml volumetric flask. Then add 0.1 *N* H_2SO_4 to volume and mix. Prepare fresh daily.

Assay Preparation—Accurately weigh about 0.5 g of pilular or powdered extract and quantitatively transfer into a 125-ml conical flask. Add 40 ml of 0.1 *N* H_2SO_4. Heat to not more than 45° and stir the mixture to hasten solution. Decant the solution through medium-porosity filter paper into a 100-ml volumetric flask. Add two successive 20-ml portions of 0.1 *N* H_2SO_4, warming each, to wash the flask and filter each through the filter paper into the 100-ml volumetric flask. Dilute to volume with 0.1 *N* H_2SO_4 and mix.

Pipet 10 ml of this solution into a 60-ml separator. To the separator, add 1.0 ml of the internal standard solution and 15 ml of chloroform. Shake vigorously, allow the layers to separate, and discard the chloroform layer. [If there is an emulsion problem, a *mixed solvent* consisting of chloroform–2-propanol (10:3) can be substituted for chloroform throughout the extraction procedure.] Add another 15 ml of chloroform and extract again, discarding the chloroform phase. Add 15 ml of pH 9.5 phosphate buffer and sufficient 1 *N* sodium hydroxide solution, dropwise, so that the final pH is 9.0–9.5. Add 15 ml of chloroform, shake vigorously, and allow the layers to separate.

Filter the organic phase through 10 g of anhydrous sodium sulfate (previously chloroform washed), supported in a funnel with a small pledget of glass wool, into a suitable container. Extract again with two successive 15-ml portions of chloroform, collecting the clarified organic phase. Then wash the bed of sodium sulfate and the tip of the funnel with 5 ml of chloroform. Rapidly evaporate[5] the combined organic phase under reduced pressure, taking care not to heat the samples to greater than 45°. Add 1 ml of chloroform and mix to dissolve the alkaloids.

Proceed with belladonna tincture following the procedure outlined for the pilular and powdered extracts. However, in preparing the assay preparation, transfer 2.0 ml of the tincture instead of "10 ml of this solution" to a 60-ml separator containing 10 ml of 0.1 *N* H_2SO_4. Record from the standard curve the quantities, in milligrams, of atropine and scopolamine in the sample. Add the quantity of milligrams of atropine and scopolamine and multiply by 50 to obtain the milligrams of alkaloids per 100 ml.

Standard Preparation—Dissolve about 10 mg of USP scopolamine hydrobromide reference standard, accurately weighed, in 0.1 *N* H_2SO_4 contained in a 10-ml volumetric flask, add 0.1 *N* H_2SO_4 to volume, and mix. Label this Solution A. Dissolve about 20 mg of USP atropine sulfate reference standard, accurately weighed, in 0.1 *N* H_2SO_4 contained in a 50-ml volumetric flask, add 2.0 ml of Solution A and 0.1 *N* H_2SO_4 to volume, and mix. Prepare fresh daily.

Standard Curve—Pipet 1.0-, 2.0-, and 3.0-ml portions of the standard preparation into three 60-ml separators, respectively, and add 9.0, 8.0, and 7.0 ml of 0.1 *N* H_2SO_4, respectively. Proceed as directed under *Assay Preparation*, beginning with " . . . add 1.0 ml of the internal standard solution . . . ."

Extraction Blank—Transfer 10 ml of 0.1 *N* H_2SO_4 to a 60-ml separator. Proceed as directed under *Assay Preparation*, beginning with ". . . and 15 ml of chloroform. Shake vigorously." The blank chromatogram should contain no significant interferences at the locus of atropine, scopolamine, or homatropine.

Procedure—Inject a portion (about 5 μl) of each standard solution into a suitable gas chromatograph equipped with a flame-ionization detector. Measure the areas, A_A, A_H, and A_S, of the atropine, homatropine, and scopolamine peaks, respectively, in each chromatogram and calculate the ratios R_A and R_S by the formulas A_A/A_H and A_S/A_H. Plot the standard curves of the values of R_A and R_S against the amounts, in milligrams, of atropine and scopolamine in the solutions.

Calculate the amounts of atropine by the formula $W_a \times (0.8329/50) \times V$ and the amounts of scopolamine by the formula $W_s \times 0.2 \times (0.6921/50) \times V$, where W_a and W_s are the weights, in milligrams, of atropine sulfate and scopolamine hydrobromide, respectively; 0.8329 is the ratio of the molecular weights of atropine to atropine sulfate; 0.6921 is the ratio of the molecular weight of scopolamine to scopolamine hydrobromide; and V is the volume of the standard preparation.

Inject a portion of the assay preparation into the chromatograph and obtain a chromatogram as on the standard solutions. Measure the peak area of the atropine, homatropine, and scopolamine[6] peaks in the chromatogram and calculate the ratios r_A and r_S by the formulas a_A/a_H and a_S/a_H. From the standard curve, record the quantities, in milligrams, of atropine and scopolamine in the volume of sample taken. Add the quantity, in milligrams, of atropine and scopolamine and multiply by 10 to obtain the milligrams of alkaloids in the sample weight.

Alternative Assay Preparation—An earlier method developed in this laboratory consisted of an extraction scheme similar to the preceding procedure. However, in the earlier method, the internal standard, homatropine hydrobromide, was extracted as the base into chloroform; at the final evaporation stage of the analysis, 1 ml of this chloroform solution was added to the nearly dry sample. Initially, the sample (1 *versus* 0.5 g) was extracted into chloroform–ethanol (3:1).

Ten milliliters of this solution (2 ml of the tincture) was transferred to a separator, the solution was acidified with 10 ml of 0.1 *N* H_2SO_4, and most of the botanical components were extracted into chloroform and discarded. The remaining solution was made basic with 1 *N* sodium hydroxide, extracted twice with two 10-ml portions of chloroform, and evaporated to near dryness. The internal standard was added, and a 5-μl sample was injected for the assay of atropine. The solution was then concentrated further to ≤0.3 ml, and 10 μl of this solution was injected for the scopolamine analysis.

RESULTS AND DISCUSSION

Several years ago, GLC methods were developed for atropine and scopolamine dosage forms (1, 2). These methods have demonstrated consistent reliability and have been extended (3) to other drug mixtures. Some investigators (6) have applied earlier GLC technology to the analysis of belladonna. Chromatographic characteristics and problems of the alkaloids now are clearly appreciated (1, 2, 6, 7), so the application of a GLC assay to the extracts seems to be in order.

The belladonna extracts of commerce are complex and not fully defined with respect to composition, thermal history, age, and exact source. There are only two regular suppliers in this country, and rather surprising differences have come to light even with the limited sampling represented by the three extracts reported here. Indeed, the initial step chosen to begin the isolation sequence far outweighs all other analytical considerations combined. The initial isolation step in the recovery of drugs (or other analyses) from complex matrixes may well be the touchstone of the analytical method, but this fact is often obscured by excessive preoccupation with the often sophisticated and expensive instrumental systems subsequently used.

General Comments—Improved official methods for the determination of total alkaloidal concentration in the extracts and tinctures of belladonna have long been desired. The present offi-

[4] Whatman 1PS.

[5] Buchner Rotary Evap-O-Mix with VacTorr 20 portable pump and dry ice trap. Evaporation time should be not more than 1 hr.

[6] For increased scopolamine precision, concentrate the assay and standard preparations to 0.3 ml and inject 10 μl.

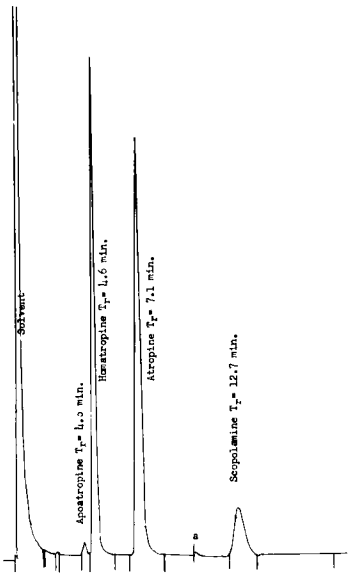

Figure 1—*Sample chromatogram of belladonna pilular extract. Key: a, a 10-fold decrease in attenuation.*

cial (4, 5) methods were in use before the 1930's and are imprecise, unspecific, and time consuming. They are based on isolation followed by acid–base titration and require multiple extractions for complete extraction of the drug. Fairly stable emulsions often are produced with each extraction step, so the extractions are unreliable and discouraging. The thermal instability of the alkaloids is well known, and breakdowns of the alkaloids by heat during this assay has been documented (6). Considering the nature of these alkaloids, the two 15-min dry-heating periods of the samples in air on a steam bath called for in the official procedure clearly can lead to substantial loss of alkaloidal content.

The isolation scheme presented here was chosen for its simplicity and similarity to the scheme used in the previously successful USP collaborative study (2) of atropine and scopolamine. The concentrations of plant materials are much lower than with the titrimetric assay procedure, so less emulsion potential exists. Two chloroform extractions from an acidified solution of the tincture and powdered and pilular extracts, as described in the *Procedure*, were sufficient to remove interfering plant materials, as shown by TLC monitoring of every individual extract along the isolation scheme.

The alkaloids were subsequently extracted into chloroform from the basified aqueous layer (pH 9.5 phosphate buffer was used instead of mineral alkali to minimize ester cleavage); 87.0% (pilular) and 87.6% (powder) of the alkaloids were recovered in the first extract, so that two additional extractions gave sufficient alkaloid recovery. The initial chloroform phases (acid extraction step) were also analyzed for atropine and scopolamine; none was found. An extraction blank is included in the procedure to monitor reagents for interfering impurities. Precision for the atropine standard carried through the procedure was 1.85% *RSD*, and chromatographic precision alone was 0.6% *RSD*. Standard curves, 0–4.5 μg of atropine, were rectilinear (correlation coefficient of 0.98, *SE* = 0.07, intercept of −0.02, based on 24 values over 14 days). A sample chromatogram is shown in Fig. 1.

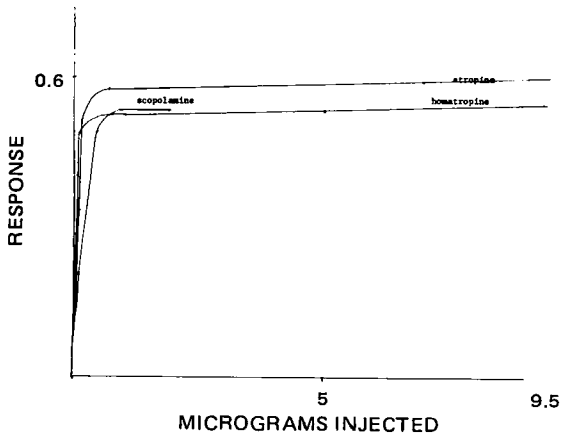

Figure 2—*Nonlinear adsorption data. Relative response times* C *of atropine, homatropine, and scopolamine* versus *anthracene: [(counts drug/counts anthracene)(weight anthracene/weight drug)] [C drug/C anthracene].*

Table I—Standard Addition Experiments

Sample	Type	Amount Added, %	Weighing	Prior Assay Value, % Atropine/ Atropine Added
I-A	Pilular	50	A	94.3, 95.6
			B	95.9, 95.9
			C	91.0, 90.1
			D	89.3, 89.3
				(average = 92.7)
I-B	Pilular	100	A	100.6, 99.8
			B	97.3, 99.2
			C	99.8, 99.8
				(average = 99.4)
I-C	Powdered	70	A	110.0, 108.0
			B	107.4, 108.8
			C	105.2, 108.4
			D	106.2, 114.1
			E	106.0
				(average = 108.2)

Nonlinear adsorption data or chromatographic recovery as a function of sample size for homatropine, atropine, and scopolamine *versus* anthracene are presented in Fig. 2. To eliminate the need for detector calibration (8), relative response values were multiplied by *C* drug/*C* anthracene, where *C* = [molecular weight/(12 × number of carbon atoms)]. Atropine and homatropine components exhibited minimal nonlinear adsorption in the region of the 3-μg sample sizes. Scopolamine recovery showed some nonlinearity and, therefore, decreased precision at its lower concentration, 0.3 μg, in the preparation used for the atropine analysis. The decreased precision in the scopolamine assay resulting from a single injection of about 0.3 μg of the drug, as opposed to ~2 μg following a concentration step and reinjection, did not substantially affect the total assay value of the belladonna extracts; therefore, the additional working time was not justifiable.

Standard addition experiments at 50, 100, and 70% of additional atropine, by pipet, with weighed amounts of previously assayed extracts resulted in recovery of added atropine of 92.7% (Lot A), 99.4% (Lot B), and 108.2% (Lot C), respectively (Table I). There still remained, however, a potential emulsion problem with the pilular extracts. This was most evident with Lot B of the pilular extract. An extraction medium of chloroform–2-propanol was substituted for chloroform throughout the procedure for this lot. As is evident, recovery using this modification was acceptable, but variable recovery due to extract lot differences is clearly indicated.

Extract Assays—When using the acid pretreatment scheme, total assay results (Laboratory I) for Supplier I were precise; the relative standard deviation values for Lots A–D were 1.1, 0.54, 0.63, and 1.02%, respectively. For Lots E and F from Supplier II, the relative standard deviations were 2.16 and 2.24%, respectively. Assay values are given in Table II. Assay values[7] for the tincture were higher than for the USP XVIII method. However, these are believed to reflect the true alkaloidal content. All samples had apoatropine contents not accounted for by injection port decomposition, probably due to the usual heat drying of the extracts during preparation. Indeed, it may develop that this content of apoatropine is an additional index of raw material quality.

Results for the Supplier II powdered extracts indicate a potential emulsion problem. A fourth extraction of the basic solution may be necessary in some cases to extract the alkaloids totally from other lots of a similar material. While complete solution in acid was evident for the pilular samples from Supplier I, such was

[7] These values were obtained before acquisition of a highly efficient vacuum pump that shortened evaporation time from several hours to less than 30 min.

Table II—Assay Results for Belladonna Alkaloids

Sample[a]	Weighing	Atropine, mg/g	Scopolamine, mg/g	Total Alkaloids[b], mg/g Average	
				Laboratory I	Laboratory II
Pilular Extract[c]					
I-A	A	12.59, 12.59	0.29, 0.31	12.81 ± 0.14	
	B	12.59, 12.65	0.29, 0.30	(*RSD* = 1.08%)	13.11
	C	12.57, 12.64	0.30, 0.30		13.63
	D	12.48, 12.44	0.29, 0.28		13.77
	E	12.25, 12.34	0.29, 0.29		
		(average = 12.51 ± 0.13, *RSD* = 1.07%)	(average = 0.29 ± 0.01, *RSD* = 2.87%).		(average = 13.50 ± 0.35, *RSD* = 2.58%)
I-B	A	11.21, 11.20	0.50, 0.50	11.71 ± 0.06	12.68
	B	11.18, 11.20	0.49, 0.50	(*RSD* = 0.54%)	12.54
	C	11.12, 11.18	0.48, 0.49		13.12
	D	11.30, 11.22	0.50, 0.49		
	E	11.30, 11.17	0.52, 0.53		
		(average = 11.21 ± 0.06, *RSD* = 0.49%)	(average = 0.50 ± 0.01, *RSD* = 2.59%)		(average = 12.78 ± 0.30, *RSD* = 2.37%)
II-E	A	11.23	0.51	11.55 ± 0.25	10.12
	B	11.07	0.58	(*RSD* = 2.16%)	11.49
	C	10.81	0.46		
		(average = 11.04 ± 0.21, *RSD* = 1.97%)	(average = 0.52 ± 0.06, *RSD* = 11.7%)		(average = 10.81 ± 0.97, *RSD* = 8.97%)
Powdered Extract[c]					
I-C	A	11.48, 11.34	0.20, 0.21	11.56 ± 0.07	11.12
	B	11.36, 11.34	0.18, 0.22	(*RSD* = 0.63%)	11.21
	C	11.36, 11.36	0.18, 0.22		11.30
	D	11.26, 11.47	0.20, 0.21		
	E	11.32, 11.28	0.22, 0.19		
		(average = 11.36 ± 0.07, *RSD* = 0.62%)	(average = 0.20 ± 0.02, *RSD* = 7.71%)		(average = 11.21 ± 0.09, *RSD* = 0.80%)
I-D	A	11.64, 11.58	0.11, 0.11	11.79 ± 0.12	11.82
	B	11.58, 11.58	0.09, 0.11	(*RSD* = 1.02%)	12.08
	C	11.74, 11.59	0.09, 0.09		12.21
	D	11.76, 11.68	0.10, 0.11		
	E	11.91, 11.80	0.12, 0.12		
		(average = 11.69 ± 0.11, *RSD* = 0.97%)	(average = 0.11 ± 0.01, *RSD* = 11.80%)		(average = 12.04 ± 0.20, *RSD* = 1.65%)
II-F	A	10.45	0.26	10.44 ± 0.23	11.16
	B	10.22	0.13	(*RSD* = 2.24%)	10.81
	C	10.15	0.12		(average = 10.99 ± 0.25, *RSD* = 2.25%)
		(average = 10.27 ± 0.16, *RSD* = 1.53%)	(average = 0.17 ± 0.08)		
		Atropine, mg/100 ml	Scopolamine, mg/100 ml		
Tincture[c]					
II-G		33.1	1.80	34.7 mg/100 ml	
		31.7	1.63		
		33.8			
		33.4			
		(average = 33.00 ± 0.93, *RSD* = 2.76%)			
USP XVIII assay		N.A.	N.A.	32.4, 31.2 } 31.8 mg/100 ml	

[a] Lots A, B, C, and D were from a single supplier; Lots E and F were from a second source. [b] All Laboratory I values are single-day, single-analyst, and single-analysis values. Second values reported are duplicate analyses of same weighing. Laboratory II conducted single determinations by two analysts on two different days. [c] Declared amounts: pilular extract, 12.5 mg/g; powdered extract, 12.5 mg/g; and tincture, 27–33 mg/100 ml.

Table III—Comparison of Assay Values for Original and Modified Methods

Extract	Code	Original Method: Weighings	Original Method: Total Alkaloids[a]	Modified Method: Weighings	Modified Method: Total Alkaloids[a]
Pilular	I-A	2	3.99	5	12.81
	II-E	5	11.78	5	11.55
Powdered	I-C	—	—	5	11.56
	II-F	3	7.08	3	10.44

[a] Average values expressed in milligrams per gram.

not the case for those from Supplier II. A single reextraction of the medium-porosity filter paper was used initially to remove the insoluble plant material, necessary to achieve total atropine–scopolamine extraction. Total assay results were still somewhat lower: 4.9% (Laboratory II) and 3% (Laboratory I), of which a 4.6% lower atropine value was noted.

An initial direct chloroform–alcohol (3:1) extraction of the alkaloids from the raw extracts was evaluated and was entirely successful for the Supplier II pilular extracts. However, when using this technique, Supplier I pilular extracts assayed at less than half the declared amount (Table III). The acid pretreatment scheme, on the other hand, yielded slightly lower Supplier II pilular extract results as compared to the organic extraction scheme. This substitution was weighed against the totally unsuccessful assay of the Supplier I pilular extracts and Supplier II powdered samples by the initial chloroform–alcohol extraction.

The identity of those solid additives necessary to produce a powdered extract needs further definition and standardization. Differences in manufacturing processes or sample constituents in the Supplier I samples clearly interfere with direct extraction with organic solvents; conversely, there appears to be similar problems with the acid extraction of Supplier II extracts, specifically lower assay values and the formation of emulsions.

Preliminary work in this laboratory included adding a portion of edetic acid during the initial part of the analysis, which increased the assay value of incompletely extracted samples by about 5%. As reported elsewhere (9), belladonna alkaloid plant material contains trace amounts of heavy metal ions[8], concentrated chiefly in the leaves. Presumably, these heavy metal contaminants plus iron from processing could be a significant source of variance between suppliers, sample extracts, but the problems encountered here are not sufficient cause to evaluate further heavy metal contamination as a possible error source.

An unsatisfactory lot of anhydrous sodium sulfate was also received in this laboratory. It lowered the assay values substantially by catalyzing on-column decomposition of the atropine samples as evidenced by the appearance of a classic decomposition in the peak envelope. Two OV-17 columns were prepared; on the first, preferential decomposition to apoatropine was observed. On the second, the atropine peak was merely severely distorted (both columns were suitable for atropine analysis). This batch of sodium sulfate did not differ from other batches in either composition or water content other than by a fourfold increase in calcium content as evidenced by X-ray methods. A reagent qualification test was developed.

[8] Heavy metal-ion contaminants include copper, molybdenum, manganese, chromium, nickel, vanadium, strontium, and barium.

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