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USP Collaborative Study of the Assay of Atropine and Scopolamine Dosage Forms

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Abstract □ Several tablet and injection dosage forms of atropine and scopolamine are covered by USP XVIII monographs. Gas chromatographic unit dose assay and content uniformity procedures were developed for these monographs. Interlaboratory reliability was evaluated by means of a collaborative study. The methods afford substantial improvements in sensitivity, specificity, and speed over previous official methods.

Keyphrases □ Atropine, scopolamine dosage forms—analysis □ Scopolamine, atropine dosage forms, analysis—collaborative study □ Content uniformity method—atropine, scopolamine dosage forms □ GLC—analysis

Previous official methods of assaying dosage forms for belladonna alkaloids have relied largely on titrimetry. These methods lacked sensitivity and specificity. Unit doses could not be assayed and decomposition products were not excluded. Such problems aroused some criticism. A notable exception was an IR method (1) which, although failing in sensitivity, did offer specificity with some control over decomposition. Assay methods for USP XVIII were desired which would be accurate, reliable, and highly specific and yet be sufficiently sensitive, precise, and rapid to allow content uniformity determinations on unit doses.

Various other approaches to belladonna alkaloid analysis may be noted. A colorimetric method (2, 3) was applied to preparations containing phenobarbital along with the alkaloids, and a dye–complex method was applied to atropine tablets and elixir (4). Neither of these approaches distinguishes one belladonna alkaloid from another. A fluorometric method has been reported for atropine (5). Paper chromatography (6, 7), partition-column chromatography (2, 3, 8), countercurrent distribution (9), TLC (10), and TLC with densitometry (11) have all achieved separation of scopolamine from atropine–hyoscyamine.

Initial efforts in the gas chromatography of belladonna alkaloids were reported by Kazyak and Knoblock (12), Brochmann-Hanssen and Fontan (13), Jain and Kirk (14), and Solomon *et al.* (15). Penner (16) studied atropine assay by GLC, both as a silyl derivative and later untreated, using tetraphenylethylene as the internal standard. Alber (17) recently reported a broad study of the gas chromatography of drugs and alkaloids using the methylphenylpolysiloxane liquid phase which was used in this collaboration.

The procedures developed for this collaborative study are related to a method previously reported (18) for dose forms of belladonna alkaloids containing phenobarbital. Other official methods[1] were developed earlier for belladonna alkaloids using anthracene as an internal standard for control of injection volume alone.

MATERIALS

Methylene Chloride—Gas chromatography or 99 mole % grade was used.

[1] Hyoscyamine sulfate tablets NF and morphine and atropine sulfates tablets NF.

Buffer—Prepare 0.2 *M*, pH 9.0 buffer, standardized against the glass electrode, by dissolving 34.8 g. dibasic potassium phosphate in 900 ml. water. Adjust to pH 9.0 and make to 1 l. with water.

Atropine Sulfate Standard Solution—Dissolve 30.0 mg. atropine sulfate in distilled water in a 100-ml. volumetric flask to obtain a solution of 0.30 mg./ml. Prepare fresh daily.

Homatropine Hydrobromide Standard Solution—Similarly, prepare a final concentration of 0.04 mg./ml. by weighing 40.0 mg. homatropine hydrobromide.

Scopolamine Hydrobromide Standard Solution—Similarly, prepare a final concentration of 0.25 mg./ml. by weighing 25.0 mg. scopolamine hydrobromide.

PROCEDURES

Atropine Sulfate Tablets—Place 1 tablet, or its equivalent from a composite of 20 tablets, in 5 ml. buffer in a 30-ml. separator and add exactly 1.0 ml. homatropine standard solution. Extract with 10 ml. methylene chloride, passing the separated organic layer through 2 g. anhydrous sodium sulfate supported by a small pledget of glass wool in a funnel. Evaporate the methylene chloride at reduced pressure to about 0.3 ml. Inject an appropriate volume, about 1 μl., into the chromatographic system. Perform the assay in duplicate. Repeat the procedure, pipeting duplicate 1.0-, 2.0-, 3.0-, and 4.0-ml. aliquots of atropine standard solution in place of the tablets.

Measure the height, *H*, of the atropine and homatropine peaks in each chromatogram and calculate $R = H$ atropine/H homatropine. Plot the value of *R* obtained from the standards *versus* amount of standard atropine sulfate added. Determine the amount of atropine sulfate in the sample preparation directly from the graph.

Atropine Sulfate Injection—Proceed as directed under tablets, substituting 1.0 ml. or the measured contents of a single-dose container of the injection for the tablets.

Atropine Sulfate Ophthalmic Solution—Pipet 1.0- or 2.0-ml. aliquots of the preparation into duplicate 50-ml. volumetric flasks and make to volume with distilled water so that the final concentration of atropine sulfate is 200–800 mcg./ml. Pipet 1.0 ml. of the sample preparation in place of the tablet, and proceed as directed in the assay for atropine sulfate tablets. Multiply the amount of atropine sulfate in the sample preparation by the dilution factor.

Scopolamine Hydrobromide Tablets—Place 1 tablet, or its equivalent from a 20-tablet composite, in 5 ml. buffer in a 30-ml. separator and add exactly 1.0 ml. atropine sulfate standard solution. Extract with 10 ml. methylene chloride, filtering the organic layer through 2 g. anhydrous sodium sulfate supported by a small pledget of glass wool in a funnel. Evaporate the solution under reduced pressure to about 0.3 ml. Inject an appropriate quantity, about 1 μl., into the chromatographic system. Perform the assay in duplicate. Repeat the procedure, substituting duplicate 1.0-, 2.0-, and 3.0-ml. aliquots of scopolamine hydrobromide standard solution for the tablet.

Measure the peak heights, *H*, of atropine and scopolamine in each chromatogram, and calculate the ratio $R = H$ scopolamine/H atropine. Plot the ratios of the standards *versus* the amount of scopolamine hydrobromide added. Read the amount of scopolamine hydrobromide in the tablet or sample preparation from the calibration graph.

Scopolamine Hydrobromide Injection—Proceed as directed in the assay for the tablets, substituting 1.0 ml. or the measured contents of a single-dose container of the injection for the tablet.

GAS CHROMATOGRAPHY

Analyses should be performed using 0.6-1.2-m. glass columns, 4 mm. i.d., packed with 3% w/w methylphenylsilicone oil[2] on 80/100- or 100/120-mesh silanized, acid-washed, flux-calcined diatomite. Flame-ionization detectors are used. Helium carrier gas is used at a flow of about 60 ml./min. The temperature of the injection port is not more than 25° above that of the column; on-column injection is preferred. Column temperature and flow may be adjusted to permit rapid (5–10 min.) and optimum analysis, about 210° for an 0.6-m. column or 225° for the 1.2-m. column.

Low-polarity methylphenylsilicone[2] is coated on silanized, acid-washed, flux-calcined diatomite. A special curing sequence has been found to increase inertness and efficiency: maintain the column at 250° for 1 hr. with helium flowing to remove oxygen and solvents, stop the flow of helium and heat at about 340° for 4 hr., lower temperature to 250°, and condition with the helium flowing until stable. A suitable initial test for support inertness, which is valuable with any low-polarity liquid phase, is the delivery of a single symmetric peak for injected cholesterol with no evidence of decomposition. The alkaloid peaks should be symmetric with little tailing.

PROTOCOL

The previously discussed details of methods and materials were supplied[3] to the collaborators along with samples of commercial dosage forms. The stated objective of the study was to evaluate these gas chromatographic methods for use in USP XVIII monographs on atropine sulfate tablets, injection, and ophthalmic solution, and on scopolamine hydrobromide tablets and injection.

Separate report sheets for each alkaloid were supplied. These requested, in addition to assay results, the identities of the instruments, support material, and supplier. Chromatographic data requested were column parameters, retention times, occurrence of peaks in reagent blanks, resolution factors (19), and efficiencies (20). The variance of an individual drug–standard ratio was to be reported for eight injections of a single sample. Procedural variance was to be estimated by 6–8-fold assay of a single bottle of an injection or of a tablet composite.

The following paragraphs were included in the protocol by way of explanation to the collaborators.

Both atropine and scopolamine are available in multiple strengths for each of the official items (tablets, injection, and solution). These procedures were prepared with this in mind and feature the use of a standard curve hinged on a single amount of internal standard. This course was chosen rather than manipulating all sample preparations to a single specified concentration,[4] because a calibration curve must be prepared in the process of approving a column for use in a single-point assay. Such a single-point approach requires a linear standard curve passing exactly through zero, an unnecessarily strict and often unattainable requirement for some drugs.

The isolation scheme is the simplest possible. Basic phosphate buffer is added so that the pH of the aqueous phase is 9 and is used instead of alkali to minimize ester cleavage. Homatropine was chosen as the "extracted" internal standard for atropine preparations for several reasons. It differs from atropine only in a methylene group and the nature of substitution of the carbinol; thus, chromatographic and chemical characteristics are similar. Multiple extractions or complete recoveries along the way are rendered unnecessary, since the molecular ratio of standard to analyte is controlled from the first step of the assay. Because of the close chemical similarities, minor alkaline ester cleavage or amine degradation also is controlled. Similarly, atropine is chosen as the standard for scopolamine. Both standards are or will be readily available. Heat and air during evaporation should be avoided.

Belladonna alkaloids are polar compounds, and the particular difficulties associated with the GLC of amines are well known. Improperly or partially cured and conditioned columns often cause extensive tailing of such compounds. An additional problem can be partial, on-column dehydration (13, 15) of atropine and scopolamine. Although the preparation of less polar derivatives may allow successful chromatography in poorer systems, the additional steps and problems are appreciable. Modern phases, supports, and column treatments have extended greatly the range of molecules that can be chromatographed directly, without prior formation of less polar derivatives. The authors had successfully determined belladonna alkaloids previously and found no reason for including derivatization in the assays. The key to this assay is in the selection of the column.

[2] OV-17. This oil contains approximately equal proportions of methyl and phenyl radicals. Other proportions are, or are becoming, available. Substitution is permitted but only if the chromatographic parameters discussed herein are met.

[3] During the summer of 1969 with all responses completed by midfall.

[4] USP XVIII since has specified a single-sample concentration but retains the calibration curve.

Table I—Assay Results: Individual Laboratory Average Assay Values for Samples Supplied

Collaborator	Replicates, n	Atropine SO_4 0.4-mg. Tab.	Atropine SO_4 0.3-mg. Tab.	Atropine SO_4 0.6-mg. Tab.	Atropine SO_4 0.4 mg./0.5 ml.	Scopolamine HBr 0.43 mg./ml.	Scopolamine HBr 0.32 mg./ml.
A	2	0.398	0.275	0.552	0.395	0.397	0.313
B	2	0.383	0.280	0.592	0.390	0.425	[0.365]
C	4–9	0.420	0.304	0.633	0.390	0.430	0.312
D	3–10	0.390	0.295	0.605	0.405	0.438	0.315
E[a]	2	0.388	0.293	0.614	0.389	0.418	0.312
F	2	0.390	0.300	0.600	0.403	0.433	0.310
G	2	0.412	0.295	0.602	0.405	0.400	0.315
H	2–4	0.407	0.265	0.566	0.370	—	—
I[b]	1	0.415	0.320	[0.510]	0.420	0.430	0.315
$\bar{x}$, mg.		0.401	0.295	0.595	0.396	0.422	0.313
$\bar{s}$, mg.		0.013	0.014	0.027	0.014	0.015	0.002
$\bar{cv}$ %		3.3	4.8	4.5	3.5	3.6	<1

[a] Diatoport S support; the others used OV-17 on Gas-Chrom Q. [b] OV-1 on Chromosorb WHP.

It was recognized that some collaborators would prefer purchasing a prepared packing. The Drug Standards Laboratory (DSL) arranged for a lot,[5] tested by DSL, to be reserved by Applied Science Laboratories, State College, Pa. The collaborators were advised that after packing the column, the described curing and conditioning were necessary.

RESULTS AND DISCUSSION

Each assay value in Table I is the average of that collaborator's individual values (as given in the second column), whether he reported duplicate or 10-fold determinations. In this way, each collaborator's result is not allowed to obscure interlaboratory variation.

Accuracy—The average assay values given in Table I are compared in Table II to the manufacturer's in-house extraction–titrimetry values determined at time of pass. The assay results by the GLC method exhibited less deviation from declared contents than the in-house extraction–titrimetry methods. On the average, the GLC values appeared to run about 0.5% lower. However, this GLC method does not measure decomposition products and should yield more realistic values.

Reference standards for each drug will now be available. What effect this standardization would have on the interlaboratory variation or on the actual assay values cannot be predicted, although some reduction in interlaboratory variation may be anticipated. With regard to assay values, results from Laboratory A, for example, averaged 3.2% below results from other laboratories, suggesting some bias in procedure, very likely related to the standards used. Laboratory H, which used a programmed temperature run, reported assay values approximately 5% lower than other laboratories. No other systematic error was apparent.

Overall, the GLC methods given here must be judged as accurate and suitable for the official USP assay methods in light of the following features.

Reliability—The data were evaluated grossly in the following manner. A mean, standard deviation, and coefficient of variation were generated for each dosage form. In four of six cases, the magnitude of the standard deviation was similar; since chromatographic properties for the two drug standard situations were essentially similar, it appears that all the data in Table I may be pooled. An average, $\bar{cv}$, of the nine coefficients of variation was obtained. The deviation of the individual assay values from the mean, $\bar{x}$, for that dosage form was considered; if $x - \bar{x} \geq 3\,(\bar{cv})\,(\bar{x})$, with $\bar{x}$ calculated without rejection, then the assay value was rejected, with the additional limitation that only one value from any column could be rejected. Only the two values in brackets out of the 52 values in Table I were rejected. This course was chosen to allow conservative estimation of reliability.

A new average of the nine recalculated coefficients of variation was then calculated to be 3.4%. This value is one estimate of the overall reliability of the methods and is composed of both intralaboratory and interlaboratory variations, as well as of significant deviations from the protocol. True reliability, of course, is a composite of this variation with accuracy, specificity, sensitivity, and precision. The contribution of each factor to the overall reliability will be discussed.

Table II—Comparison of Assay Values

Dosage Form	Manufacturer	USP Collaborative
Atropine Sulfate Tablets		
0.4 mg.	94.0%	100.0%
0.3 mg.	95.0	98.4
0.6 mg.	97.3	99.2
Atropine Sulfate Injection		
0.4 mg./ml.	103.6	99.0
Scopolamine HBr Injection		
0.43 mg./ml.	101.0	98.4
0.32 mg./ml.	100.4	98.0
Average	99.7	99.2

Specificity and Sensitivity—The inherent specificity and sensitivity of gas chromatography, combined here with an extracted internal standard, are utilized by these methods. In addition to serving as the monograph assay, the comparison of the sample chromatogram to that of the reference standard serves also as a strong identity test. Degradation products do not interfere. Tropic acid is not extracted in the first step, and tropine and scopine elute prior to the standards and drugs. No collaborator reported evidence of decomposition products.

The methods are directly applicable to available strengths of dosage forms covered by the monographs identified in the object of the collaborative study. Indeed, only a fraction of a percent of the available sample is actually analyzed. Use of this sensitivity in proof of content uniformity of tablets is obvious; however, this application depends also on intralaboratory chromatographic precision.

Precision—Collaborators supplied data for both the reproducibility of the ratio obtained upon multiple injection of a single drug-

Table III—Precision Data Presented as Coefficients of Variation

Laboratory	Atropine R	Atropine R, %	Atropine Assay	Scopolamine R	Scopolamine R, %	Scopolamine Assay
A[a]	1.58	0.47	0.87	1.37	1.31	1.48
B	0.49	2.81	5.56	0.32	2.93	—
C	0.97	0.57	—	0.43	0.62	—
D	0.52	0.6	0.6	0.45	1.0	2.2
E[b]	0.89	0.65	0.79	0.80	0.68	0.99
F	1.07	0.65	2.92	1.06	1.04	1.24
G	1.10	0.73	—	1.01	0.82	0.65
H	1.11	2.30	3.43	—	—	—
I[c]	1.19	3.2	2.7	1.19	3.2	2.7

[a] Electronic integrator. [b] Diatoport S support. [c] Disk integrator, also used OV-1/Chromosorb WHP instead of recommended packing; all others used OV-17 on Gas-Chrom Q.

[5] OV-17 on Gas-Chrom Q.

Table IV—Chromatographic Data

Laboratory	Retention Time (Atropine), min.	Resolution Factor: Homatropine/ Atropine	Resolution Factor: Atropine/ Scopolamine	Plates: Homatropine	Plates: Atropine	Plates: Scopolamine	Column, m.
A	5, 4	—	—	1450	1270	1420	1.2
B	11	3.0	4.3	470	600	760	0.9
C	4.9, 4.6	—	>20	(2680)	1550, 1200	1390	(0.6)
D	3.7	3.6	4.8	960	1080	1140	0.6
E	5.5, 3.5	3.8	3.7	620	690, 430	570	0.55
F	4.9, 4.3	—	—	950	1360	1580	1.2
G	4.3	3.5	4.9	1010	1480	1520	1.2
H	4.5[a]	6.0	—	—	—	—	1.2
I	5, 4	1.7	1.8	1080	1420	1530	2.0

[a] Programmed run, 135–210° at 10°/min.

standard mixture and of the ratio obtained from multiple assay of a single commercial item. Their results are found in Table III. The directions are for peak height ratios; however, two laboratories used area-measuring devices.

Laboratory H erroneously did a programmed 10° per minute run, and the degraded precision is, therefore, understandable. Laboratories B and F generated a variable in obtaining sample preparations which F did not carry over from atropine to scopolamine. Otherwise, the assay manipulations appear to contribute little to (im)precision which, therefore, must be largely dependent on chromatographic precision. An approximation of the difference in the two sets of precision data is about 0.5% in coefficient of variation. Indeed, any significant departure from this level should signal a possible procedural error to the analyst.

Without rejecting any value, the average of coefficients of variation in R is 1.3% for atropine and 1.4% for scopolamine, with a further estimate of about 1.9% for dosage form assay, which is moderately good for a GLC method. Several values can be accounted for by graphical error alone. This is completely satisfactory (21) for use in content uniformity testing.

The collaborative procedures construct calibration curves for the alkaloids with four points for atropine and three points for scopolamine, based on the range of anticipated concentration of sample preparations from the range of dose strengths. Nonlinear absorption, tailing, and relative graphical error could be expected to cause somewhat greater imprecision in the lower values on the calibration curve than in the higher values. These factors would similarly affect precisions for the absolute sizes of samples injected. A plot of the percent coefficient of variation against the value of R for each alkaloid, as given in Table III, failed to support any such correlation between precision and magnitude of R. Therefore, the precision discussions in this report must pool all values irrespective of magnitude or the identity of the drugs. Variations in sample size injected and magnitude of R conversely cannot be cited as causes of interlaboratory variations. This also is consistent with the judgment that reference standards are the only further vehicle needed to control interlaboratory variation in these assays.

Dosage Forms—The 0.4-mg. atropine sulfate tablets were compressed tablets; the other two were hypodermic tablets. The results show the method to be equally applicable. Although the protocol gave a separate procedure, scopolamine hydrobromide tablets were not issued to collaborators. The atropine sulfate ophthalmic solutions available are sufficiently concentrated to require an initial 25- or 50-fold dilution to enter the assay concentration range. Thus, samples were not issued to collaborators. Results in this laboratory have shown that the procedure works as well for the nonofficial ophthalmic solutions containing cellulose derivatives.

General Observations—Intralaboratory precision was found to be primarily a matter of chromatographic reproducibility. It would appear worthwhile to prepare a packed column giving good performance, particularly where use is anticipated in content uniformity testing. The collaborators' data are of some guidance here.

The chromatographic parameters reported by the collaborators are given in Table IV. A minimum resolution of R, not less than 3.0, is one reasonable standard which an analyst might apply to a column. Laboratory I reported much lower resolution (Table III) and experienced poor precision; both results may reflect the use of a different (OV-1) phase. The authors' initial studies (18) leading to the choice of packing would support this conclusion. Similarly, efficiencies of the order of 1000 plates for any peak appear suitable, but much less efficiency can be tolerated, as shown by Laboratory E using a 0.6-m. column. Some of the reported efficiencies appear in error for the claimed column lengths. Most collaborators reported linear standard curves with the intercept near or at the origin.

However, perhaps the parameter most predictive of precision would be tailing. The authors found that tailing factors[6] larger than 1.6 correlated with diminished precision and, for this reason, the protocol sent to collaborators stated that peaks should be symmetric with little tailing.

Laboratory H erroneously used a programmed temperature run. This commonly degrades both precision and accuracy,[7] and the authors are strongly against this unnecessary operation. Speed also is lost.

The collaborators offered several comments on procedure. Laboratory H found the concentrates following evaporation were stable for 1 or 2 days. The authors made similar observations but do not recommend unnecessary sample storage. Laboratory H also reported that initial experiments indicated that evaporation at 60° in an airstream was satisfactory.

Laboratory A reported an extraneous peak in the homatropine and scopolamine standards; the homatropine appeared to contain 1.5% atropine. Such problems are best avoided by reference standards.

No collaborator reported evidence of decomposition, probably because the alkaloids were injected as free bases at moderate temperatures, with injection block temperatures only slightly greater than on-column temperatures. There have been reports (15, 22) of additional injection-site decomposition, possibly related to the glass wool at the top of the column or the injection of salts. The authors used commercial silanized glass wool and did not observe decomposition.

SUMMARY

The gas chromatographic assay methods for atropine sulfate tablets, injection, and ophthalmic solution and for scopolamine hydrobromide tablets and injection, now covered by USP XVIII monographs, have been studied in a nine-laboratory, collaborative study. The drug is extracted once from alkaline buffer along with added internal standard, homatropine for atropine and atropine for scopolamine. The extract is concentrated and injected into a defined system without further treatment. The methods are accurate, reliable, sensitive, highly specific, rapid, and reasonably precise. The methods are suited to content uniformity testing and serve as strong identity tests for all these monographs. Some interlaboratory variations suggest the need for alkaloid reference standards keyed to these methods to control systematic error. No changes in the procedures were found necessary.

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[7] The protocol uses peak heights; automatic peak area methods would be expected to maintain quantitative value.

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Determination of Total Iron in Hematinics by Atomic Absorption Spectrophotometry

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Abstract □ The total iron content of six hematinic preparations was determined rapidly, precisely, and accurately by atomic absorption spectrophotometry. Hematinics comprising iron–carbohydrate complexes required ashing prior to assaying by atomic absorption spectrophotometry while those with an iron chelate or simple salt structure may be determined directly by atomic absorption spectrophotometry. A statistical evaluation of the data indicated that the atomic absorption spectrophotometry method was equivalent to the official colorimetric and volumetric methods and to a classical gravimetric procedure.

Keyphrases □ Iron in dosage forms—analysis □ Atomic absorption spectroscopy—analysis □ Colorimetric analysis—spectrophotometer □ Titration—iron analysis □ Gravimetric analysis—iron

Hematinic preparations generally fall into three structural categories: iron–carbohydrate complexes, iron chelates, and iron salts. Preparations consisting of iron–carbohydrate complexes are usually assayed for total iron by a lengthy colorimetric (1) or gravimetric procedure.[1] The usual USP (2) or NF (3) procedure for determining the total iron content of iron chelates and iron salts involves a sodium thiosulfate or ceric sulfate titration. Extensive studies (4, 5) indicate that atomic absorption spectrophotometry (AAS) offers a technique for assaying iron which is relatively free from interfering ions. To date, no studies have been reported in the literature concerning the assay of total iron in hematinics by AAS.

[1] The procedure used in this study was a slight modification of the gravimetric iron assay procedure described in most quantitative analysis textbooks. (See H. H. Willard, N. H. Furman, and C. E. Bricker, "Elements of Quantitative Analysis," 4th ed., D. Van Nostrand, Princeton, N. J., 1956, pp. 335, 336.)

EXPERIMENTAL

Instruments—A Perkin-Elmer model 303 double-beam spectrophotometer, equipped with an iron hollow cathode lamp and single-slot burner head, was used for all atomic absorption measurements. The instrument was optimized with a 10-p.p.m. standard iron solution. A sensitivity of 0.18 mcg./ml. for 1% absorption was achieved. Instrument parameters appear in Table I. All colorimetric measurements were carried out on a Perkin-Elmer model 202

Table I—Instrument Parameters

Wavelength	248.3 mμ
Hollow cathode lamp current	30 ma.
Fuel	Acetylene (flow meter at 9)[a]
Oxidizer	Air (flow meter at 9)[a]
Aspiration rate	1.8 ml./min.
Slit	No. 3
Meter response	No. 2
Recorder	Perkin-Elmer model No. 165

[a] Perkin-Elmer Burner Control Box No. 303-0240.