GLC Analysis of Chloramphenicol: A Collaborative Study

MICHEL MARGOSIS

Abstract □ A GLC method for the assay of chloramphenicol was subjected to a rigorous collaborative study. An overall recovery of 100.1 ± 2.97% for three samples from 14 participating laboratories was obtained.

Keyphrases □ Chloramphenicol—collaborative GLC analysis in 17 laboratories □ GLC—analysis, chloramphenicol, collaborative study in 17 laboratories

The GLC method for the assay of chloramphenicol in pharmaceutical products reported previously (1) included the separation of the drug and was shown to be more specific, precise, accurate, and reliable than the current official UV and microbial assay methods (2). However, to demonstrate and validate its usefulness in other laboratories, it appeared advisable to subject this method to a wide collaborative study. The intent was to devise an analytical system that would maintain control while allowing maximum flexibility so that the method could be performed anywhere by any competent analyst.

COLLABORATIVE STUDY

Separate vials containing three "unknown" samples, two bulk materials, and one internal standard (*m*-phenylene dibenzoate) were prepared and sent to 22 laboratories[1]. The first unknown sample contained a dried mixture of 91.83% chloramphenicol and 8% of chloramphenicol palmitate; unknown samples 2 and 3 were commercial capsule granulations containing 71.90 and 64.89% chloramphenicol, respectively. The nominal values of the chloramphenicol concentration in these mixtures were obtained from the manufacturer and verified independently by this GLC procedure and by the official UV method. One bulk material was provided for use as a reference standard, and the other was provided to allow the analyst to become familiar with the method and to optimize the instrumental conditions.

EXPERIMENTAL

A final protocol of analysis for chloramphenicol by GLC was sent to each laboratory.

Materials—*Gas Chromatograph*—The gas chromatograph, capable of accommodating a glass column and attaining temperatures of 350°, was equipped with a flame-ionization detector. The injection and detector temperatures should be the same as or above that of the column (ranging from 190 to 240°, depending upon the variables in individual instrument systems) but not exceed it by more than 15°.

Column—A glass column, 0.9–1.9 m (3–6 ft), 4 mm i.d., was packed with 1–5% (w/w) low polarity methyl silicone gum or fluid on 80–100- or 100–120-mesh silanized, acid-washed, flux-calcined diatomite[2]. Apply carrier gas at ambient temperature for about 15 min; then condition with no flow for 1 hr at 340° and then with a flow of carrier gas at isothermal operating temperature until stable. It may be further sample-conditioned with a silanizing and column conditioning agent[3] to minimize adsorption. On-column injection is recommended if available. *Caution:* Use a minimum amount of silanized glass wool for plugging the ends of the column, because it may have a deleterious effect on the analysis.

Subsequent to column curing and conditioning, perform a suitability test for inertness of support by injecting cholesterol. A single, symmetrical peak with no evidence of decomposition should be obtained. Typically, the 0.9-m (3-ft) column with 3% OV-1 on 100–120-mesh Gas Chrom Q used for the analysis of these collaborative samples gave about 1200 apparent plates (or about 1350 plates/m), with a tailing factor of 1.29 for cholesterol.

The use of a low polarity dimethyl silicone polymer such as OV-1, OV-101, or SE-30 is recommended. The relative retention time factor for chloramphenicol *versus* *m*-phenylene dibenzoate (internal standard) on OV-1 (0% phenyl) is 2, whereas it is 4 on OV-17 (50% phenyl) and about 11 on PPE-20 (poly-M-phenyl ether).

Detector—Flame ionization, with air and hydrogen flow rates adjusted so as to obtain maximal response, is used. The flow rate of the carrier gas, helium, is about 60 ml/min. Adjust the column temperature and carrier gas flow rate so as to offer complete resolution of the peaks (about 5 min from solvent to chloramphenicol). Set current at 2×10^{-9} amp full-scale deflection (fsd), or adjust to obtain peak heights greater than 50% fsd, depending upon sharpness of peak.

Reagent Solution (Prepare in Hood)—Dissolve about 200 mg of *m*-phenylene dibenzoate in about 6 ml of acetonitrile. Add 1 ml of *N,O*-bis(trimethylsilyl)acetamide, and dilute to 10 ml with acetonitrile with shaking until a uniform phase is obtained. (See *Discussion.*)

Sample Preparation—*Bulk and Standard*—Accurately weigh 5–10 mg of the specimen directly into a reaction tube (conical centrifuge tube), or make a stock solution in ethyl acetate, transfer an aliquot equivalent to 5–10 mg to the tube, and evaporate to dryness under a current of dry air.

Dosage Forms—Accurately weigh the sample and make a solution in ethyl acetate. Allow insoluble excipients to settle, transfer an aliquot equivalent to 5–10 mg, and evaporate to dryness.

Procedure—To each dried sample, add 1.00 ml of reagent solution and stir vigorously[4] to obtain a single uniform phase. Inject 1 μl (equivalent to 5–10 μg of chloramphenicol) into the gas chromatograph. Retention time for bis(trimethylsiloxy)-chloramphenicol should be no less than 4–5 min. Measure the *area* of each peak by a suitable nondestructive technique.

Calculations—Equation 1 was used:

$$\%\ (w/w)\ \text{chloramphenicol} = \frac{R_{sa}}{R_{std}} \times \frac{W_{std}}{W_{sa}} \times 100 \quad \text{(Eq. 1)}$$

where R = ratio of bis(trimethylsiloxy)-chloramphenicol to inter-

[1] The 17 that actually participated are listed here. The author acknowledges the gracious participation of the following collaborators: D. H. Calam, National Institute for Medical Research, London, England; Vernon G. Davies, Parke-Davis Laboratories, Detroit, Mich.; Roberta Hartman and Joy Tagle, McKesson Labs., Bridgeport, Conn.; Gerard Janssen and Hubert Vanderhaeghe, Katholieke Universiteit te Leuven, Belgium; Siv Johannson and Bengt Ohrner, Apotekens Centrallaboratorium, Solna, Sweden; Ernest J. Kubiak and Marvin Grostic, The Upjohn Co., Kalamazoo, Mich.; Dr. Lettenbauer and Dr. Futscher, Boehringer Mannheim Gmbh, Germany; A. M. Monard, Association Pharmaceutique Belge, Service de Controle des Medicaments, Brussels, Belgium; Ida Mortensen and Jørgen Bang, Statens Serumsinstitut, Copenhagen, Denmark; A. Pacheco, Zenith Laboratories, Northvale, N.J.; Robert Puchalski, S. B. Penick and Co., Lyndhurst, N.J.; Bruce A. Ross, Food and Drug Administration, Office of Pharmaceutical Research and Testing, Division of Drug Chemistry, Washington, D.C.; Don Rowe, Food and Drug Administration, Office of Pharmaceutical Research and Testing, National Center for Antibiotic Analysis, Washington, D.C.; H. Van der Veen, Vrije Universiteit, Amsterdam, The Netherlands; Clyde E. Wells, Food and Drug Administration, Office of Pharmaceutical Research and Testing, National Center for Drug Analysis, St. Louis, Mo.; William L. Wilson, Food and Drug Directorate, Ottawa, Ontario, Canada; and Dorothy K. Wyatt, Drug Standards Laboratory, Washington, D.C.

[2] A 0.91-m (3-ft) × 4-mm column with 3% OV-1 on 100–120-mesh Gas Chrom Q (Applied Science Laboratories, Inc.) is recommended.

[3] Silyl-8, Pierce Chemical Co., Rockford, Ill.

[4] With Vortex mixer.

Table I—Results for Chloramphenicol Bulk (Sample 1)

Laboratory	Number of Runs	Mean Recovery, %	Coefficient of Variation
1	4	100.07	1.07
2	4	106.09	2.56
3	8	102.70	3.10
4	4	101.34	1.14
5	4	99.16	0.48
6	4	100.92	0.83
7	2	98.11	0.37
8	2	100.94	1.14
9	4	100.81	4.33
10	4	106.04	8.39
11	2	101.33	0.08
12	4	102.22	1.53
13	2	102.47	1.35
14	2	102.20	0.51
15	2	99.19	1.12
16	4	100.42	1.31
17	8	99.26	2.74
WD Mean	64	101.44	3.43
Mean	17	101.37	2.16
Corr Mean	14	100.85	1.35

Table II—Results for Chloramphenicol Capsule Granulation (Sample 2)

Laboratory	Number of Runs	Mean Recovery, %	Coefficient of Variation
1	8	100.59	0.96
2	4	104.24	1.26
3	8	105.41	2.10
4	4	101.32	1.14
5	4	99.22	0.97
6	4	88.94	6.49
7	2	97.28	0.78
8	2	99.06	3.58
9	4	97.73	2.28
10	4	101.82	8.28
11	2	101.39	0.58
12	4	101.67	1.01
13	2	102.71	1.24
14	2	103.67	0.08
15	2	100.54	1.54
16	4	97.84	1.20
17	8	106.29	4.18
WD Mean	68	101.16	4.98
Mean	17	100.57	3.96
Corr Mean	14	99.81	3.91

nal standard peak, and W = weight of standard or sample (std or sa, respectively).

Collaborators were requested to run duplicate injections of two separate weighings of each material and to compare the values to the average value obtained on the standard; they were also requested to determine the coefficient of variation of five injections of each standard. To ascertain the suitability of experimental conditions of the chromatographic system, a measure of efficiency, resolution factors, and tailing factors was also requested:

$$\text{efficiency} = 16(tr/Wb)^2 \qquad \text{(Eq. 2)}$$

where tr = adjusted retention time, and Wb = peak width (width at base cut by the two tangents drawn to the inflection point of the peak);

$$\text{resolution factor} = \frac{2D}{A + B} \qquad \text{(Eq. 3)}$$

where D = distance between peak maxima, A = Wb for peak 1, and B = Wb for peak 2; and:

$$\text{tailing factor} = \frac{A + B}{2A} \qquad \text{(Eq. 4)}$$

measured at 5% of peak height where A = distance from time point of peak maximum to ascending slope, and B = distance from time point of peak maximum to descending slope.

RESULTS AND DISCUSSION

Statistics—All calculations, data processing, and statistical analyses were performed through the APL time-share system of a computer[5]. Calculations and results from each collaborator were verified when sufficient data were available. The final results were normalized to the nominal concentration of the sample as described initially and are summarized in Tables I–IV for each sample and for the sum of the three samples. In each of the four tables, "WD Mean" is that obtained through the weighted analysis of all observations from each collaborator, inclusive of "within sample" errors; "Mean" is that obtained from the calculated mean from each collaborator, thus attributing equal weight to each collaborator. As shown in these tables, mean percent recoveries of 101.37 ± 2.16, 100.57 ± 3.96, and 100.4 ± 3.68 were obtained for Samples 1, 2, and 3, respectively, with an overall recovery for all samples from all 17 collaborators of 100.67 ± 3.32%. Although these recoveries amply validate the method and demonstrate its reliability, results from three collaborators were rejected outright. Those of two collaborators (10 and 17) were rejected because a precision exceeding 8% in the replications of the standard was obtained; results from Collaborator 2 were rejected because they exceeded an acceptable sum rank limit by the Youden (3) test. The final overall recovery for all samples from 14 collaborators was 100.1 ± 2.97%.

A computer plot of all available values *versus* the frequency of observations, numbering 190, yielded the typical S-shaped curve. A chi-square test fitted these data to a normal distribution as expected. Data obtained by electronic and disk integration were analyzed by Bartlett's test; it showed that the variances within each sample were homogeneous, indicating similar reliability from each collaborator. Data obtained by the peak height method most adversely affected this homogeneity of variances.

Duncan's multiple-range test was used to determine which collaborators differed from each other at a particular level of significance. At $p < 0.05$, overlapping random groups of three to seven collaborators showed no significant difference *within* groups of collaborators, but significant differences were noted among these same groups. With Sample 2, results of Collaborator 6 were most significantly different from all others; with Sample 3, results of Collaborators 3 and 6 were significantly different from each other and from those of all other collaborators.

A one-way analysis of variance was performed on the results calculated from each sample. At $p < 0.05$, significant differences between collaborators were detected for Samples 2 and 3 but not for Sample 1. Variations between replicate samples in all cases were fairly small or actually negligible. Differences among laboratories were found to be largely attributable to results derived by manual techniques of peak measurements. A two-way analysis of variance of the means (for each sample from each collaborator) showed no statistical differences between samples at $p < 0.05$ but confirmed the differences among laboratories as noted previously.

Methodology—Thirteen different models of GLC instruments were used, with a flame-ionization detector being about the only common denominator among these. Deviations between analysts could be ascribed largely to differences in such characteristics as nature (type and density) of packing, carrier gas, pressure drop, column material, length, diameter, and efficiency. The columns ranged in length from 0.91 to 1.83 m (from 3 to 6 ft), and various supports were employed under different conditions of temperature and carrier gas flow rates. Most used helium, but some used nitrogen.

To maintain control of this method, it is essential to ascertain that a minimum performance requirement be met *a priori* for the entire analytical system whatever components it may include. In the case of inert supports, such as the silicones, a curing, conditioning, and suitability test similar to that described in USP XVIII should become mandatory. A failure of this test probably indicates an inadequate packing material. In all cases, a mini-

[5] IBM 360.

Table III—Results for Chloramphenicol Capsule Granulation (Sample 3)

Laboratory	Number of Runs	Mean Recovery, %	Coefficient of Variation
1	4	100.72	1.35
2	4	108.53	1.34
3	6	105.56	1.82
4	4	101.41	0.33
5	4	99.07	0.20
6	4	93.00	0.10
7	2	97.48	0.28
8	2	99.70	0.80
9	4	96.00	1.22
10	—	—	—
11	2	102.10	0.11
12	4	99.45	0.84
13	2	101.86	0.86
14	2	100.14	0.50
15	2	97.67	0.98
16	4	96.86	1.74
17	8	101.04	1.99
WD Mean	58	100.34	3.98
Mean	16	100.04	3.68
Corr Mean	14	99.36	3.10

Table IV—Combined Results for Three Chloramphenicol Samples

Laboratory	Number of Runs	Mean Recovery, %	Coefficient of Variation
1	16	100.49	1.09
2	12	106.29	2.38
3	22	104.46	2.67
4	12	101.36	0.86
5	12	99.15	0.58
6	12	94.29	6.39
7	6	97.62	0.57
8	6	99.90	1.91
9	12	98.18	3.41
10	8	103.93	8.02
11	6	101.61	0.46
12	12	101.11	1.63
13	6	102.35	0.98
14	6	102.00	1.59
15	6	99.13	1.61
16	12	98.37	2.06
17	24	102.20	4.24
WD Mean	190	101.00	4.21
Mean	50	100.67	3.32
Corr Mean	42	100.01	2.97

mum efficiency, resolution, and symmetry factor should be specified, as well as a maximum coefficient of variation of a certain number of replicate injections of a standard, before the system is deemed acceptable for actual analysis.

The efficiencies of the columns used in this study ranged from 840 to 1580 theoretical plates/m, with a mean of 1140 ± 20% theoretical plates/m. The symmetry or tailing factor ranged between 1.04 and 1.3, with most at about 1.1. The retention time of the bis(trimethylsiloxy)-chloramphenicol relative to the internal standard is also used as a qualitative tool and is particularly valid when related to an authentic sample. In this study, the relative retention times of bis(trimethylsiloxy)-chloramphenicol ranged between 1.76 and 2.04, with a mean of 1.88 ± 3.3%.

Derivatization—This was probably the most common source of difficulty in the analysis. Several collaborators reported the presence of one or two extraneous peaks when using *N,O*-bis(trimethylsilyl)acetamide as the reagent as specified. These peaks have been ascribed to the monotrimethylsilyl and tris(trimethylsilyl) derivatives (5) and were recently corroborated in this laboratory with an OV-17 column, which allows greater resolution than the nonpolar OV-1 phase prescribed for this analysis. Furthermore, it was noted that not only did *N,O*-bis(trimethylsilyl)-acetamide behave in this manner but so did the trifluoro reagent. These occurrences were not constant; they appeared with some lots of reagents and not others. The causal factors, whether they be due to extraneous silyl donors present, the age of the lot, storage, and/or reaction temperatures, *etc.*, could not be ascertained even by the manufacturer of this reagent. Several users, however, tend to ascribe this difficulty to the age of the reagent.

It is advised, therefore, that should the reagent yield more than one major peak, alternative reagents, such as hexamethyldisilazane combined with trimethylchlorosilane, Tri-Sil, or trimethylsilylimidazole (specific for hydroxyl functions), that will result in obtaining the proper bis(trimethylsilyl) derivative be employed.

GENERAL NOTES

Comments were received from collaborators; some are useful in optimizing the method for more widespread applicability. Some, discussed randomly here, have been incorporated into the method.

1. Although helium was specified as carrier gas, other gases (*e.g.*, nitrogen) may be used if proven to be satisfactory.

2. *m*-Phenylene dibenzoate may be slow in dissolving initially or in redissolving from low temperature storage; however, this is easily remedied with gentle heating and mixing.

3. It is preferable to complete the analysis within the day of silylation to minimize any change of configuration.

4. It is easier and more accurate to take an aliquot of 1.00 ml of reagent than the 0.5 ml prescribed in the procedure.

5. The final concentration and amount injected could be reduced to avoid overloading the column. The amount of bis(trimethylsiloxy)-chloramphenicol can easily be reduced two- to 10-fold without adversely affecting the accuracy and precision of the system; in fact, this should improve the efficiency, resolution, and symmetry factors. Naturally, this also depends to some extent upon the quality of the instrument employed.

CONCLUSION

These results indeed prove the merits of this method. Because of its demonstrated superiority, it has been proposed for inclusion into the *Code of Federal Regulations* and is recommended for primary compendial usage.

REFERENCES

(1) M. Margosis, *J. Chromatogr.*, **47**, 341(1970).

(2) Code of Federal Regulations, Title 21, Part 141d, 1971.

(3) W. J. Youden, "Statistical Techniques for Collaborative Tests," Association of Official Analytical Chemists, Washington, D.C., 1967.

(4) "The United States Pharmacopeia," 18th rev., Mack Publishing Co., Easton, Pa., 1970, p. 794.

(5) G. Janssen and H. Vanderhaeghe, *J. Chromatogr.*, **82**, 297(1973).

ACKNOWLEDGMENTS AND ADDRESSES

Received August 22, 1973, from the *National Center for Antibiotic Analysis, Food and Drug Administration, U.S. Department of Health, Education, and Welfare, Washington, DC 20204*

Accepted for publication October 4, 1973.

The author thanks Ms. Janet A. Springer, Food and Drug Administration, Division of Mathematics, Bureau of Foods, Washington, D.C., for helpful discussions on, and assistance with, statistics.