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ANALYSIS OF ANTIBIOTICS BY GAS CHROMATOGRAPHY

II. CHLORAMPHENICOL

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SUMMARY

Chloramphenicol in pharmaceutical products is determined by a gas–liquid chromatographic (GLC) method which involves the formation of the ditrimethylsilyl ether with BSA prior to chromatographic injection, and normalization of the response with *m*-phenylene dibenzoate as the internal standard. The method has been applied successfully to bulk material as well as to some pharmaceutical dosage forms. Results obtained by GLC compare favorably with those obtained by official assay methods.

INTRODUCTION

Chloramphenicol, D(—)-*threo*-*p*-nitrophenyl-2-dichloroacetamido-1,3-propanediol, is an antibiotic originally obtained by fermentation from *Streptomyces venezuelae*, but is currently produced synthetically.

Many methods are available for the assay of chloramphenicol, among which are the microbiological[1], polarographic[2], and spectrophotometric either in the UV directly[1], or in the visible region after some color formation[3–5]. Logically the microbiological assay should be preferred since the end use activity of the product is the one function measured directly. However, improvement in precision, specificity, and rapidity of this method would be desirable for the analytical control of this drug. On the other hand, the other assay methods rely inherently on some particular functional groups which might also be present in contaminants or degradation products that may be biologically inactive yet contribute to the total assay results[6].

Gas–liquid chromatographic procedures are more specific, precise, and accurate than biological assays. GLC methods have been reported for the analysis of chloramphenicol, as the trimethylsilyl ether, in biosynthetic studies[7] and in biological fluids[8]. However, the application of gas chromatographic analysis to chloramphenicol without derivatization[9] has certain drawbacks, as previously discussed[10].

This paper presents and discusses the analysis of chloramphenicol in bulk and in some pharmaceutical dosage forms by gas–liquid chromatography after silylation with N,O-bis(trimethylsilyl)-acetamide (BSA).

EXPERIMENTAL

Apparatus

A Barber-Colman Model 5000 gas chromatograph with a flame ionization detector was used, with a 5-mV range recorder and 1/3 in./min chart speed. Column: glass, U-shaped, 6 ft. × 3 mm I.D., packed with 5 % SE-30 on Gas-Chrom Q (80–100 mesh). Temperatures: column, 240°; detector, 250°; injector, 250°. Carrier gas: nitrogen at 20 p.s.i. (50 ml/min), hydrogen at 32 p.s.i., and air at 40 p.s.i. (adjusted for maximum response). Current: 5×10^{-8} A f.s.d. (sensitivity 100, attenuation 5).

Reagents

Solvents. The following solvents were used: anhydrous methanol, reagent grade; ethyl acetate and acetonitrile, glass distilled; diethyl ether, reagent grade; approximately 0.01 *N* HCl.

Reagent solution. Two hundred milligrams of *m*-phenylene dibenzoate are dissolved in about 6 ml of acetonitrile. One milliliter of N,O-bis(trimethylsilyl)-acetamide (BSA) is added and the solution is made to 10 ml with acetonitrile and shaken until uniform.

Chloramphenicol standard. About 125 mg of the standard is accurately weighed into a 25-ml volumetric flask, dissolved with ethyl acetate (or methanol), and made to volume. A 2-ml aliquot is transferred to a conical centrifuge tube and is evaporated to dryness on a steam bath under a current of dry air or nitrogen.

Sample preparation

Bulk materials. Samples are prepared in the same manner as the chloramphenicol standard above.

Capsules. The empty capsules and their contents are placed in a volumetric flask, shaken vigorously with ethyl acetate (or methanol), made to volume, and allowed to settle or filtered if necessary. An aliquot of the supernatant (or filtrate) containing about 10 mg of chloramphenicol is transferred to a centrifuge tube and evaporated to dryness as above.

Tablets. A number of tablets are ground in a mortar under a few milliliters of ethyl acetate, quantitatively transferred to a volumetric flask with the aid of ethyl acetate, made to volume, and subsequently prepared like the capsules, above.

Procedure

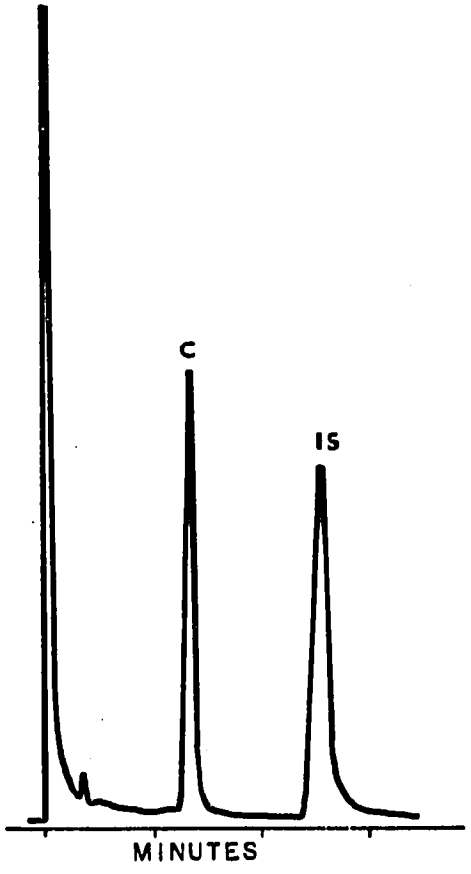
Solutions. A known volume of the preparation is transferred to a separatory funnel and diluted at least five-fold with water. This solution is extracted at least three times with a mixture of ethyl acetate–diethyl ether (2:1). Each extract is backwashed with an aqueous solution, combined, evaporated to dryness, and reconstituted to a known volume with ethyl acetate in a volumetric flask. An aliquot containing an estimated 10 mg of the drug is transferred to a centrifuge tube and evaporated to dryness on a steam bath under a current of dry air or nitrogen.

Ointments. A mixture of about 3 g of Celite 545 (acid-washed) and 2 ml of a pH 5.80 phosphate buffer is placed directly over a small wad of glass wool in a partition column tube and tamped gently to a uniform mass. A mixture of 3 g of Celite and about 2 g of the ointment (accurately weighed) is placed on top and again tamped

gently. About 100 ml of cyclohexane are passed through this column and discarded. The chloramphenicol is then eluted with about 100 ml of ethyl acetate, and the eluate is collected, evaporated to dryness, and reconstituted to a known volume with ethyl acetate. An aliquot containing an estimated 10 mg of chloramphenicol is transferred and evaporated to dryness as above.

Derivatization. To each dried sample (and standard), 0.50 ml of the reagent solution is added and stirred vigorously with a Vortex mixer to obtain a single uniform phase.

Chromatography and calculations. One microliter of each derivatization solution (equivalent to about 20 μg of chloramphenicol) is injected into the gas chromatograph. The area of each peak is measured by a suitable technique. Chloramphenicol content is determined by direct comparison of the ratio of the peak areas (chloramphenicol/internal standard) with that of the chloramphenicol standard treated in an identical manner.



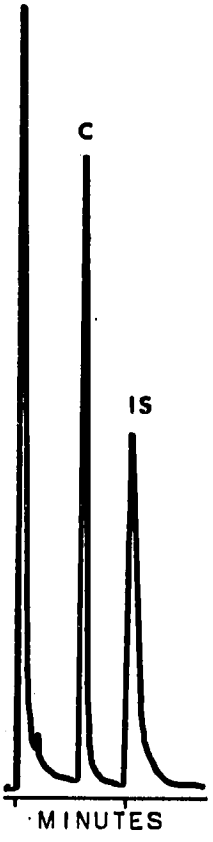


Fig. 1. Gas chromatogram of chloramphenicol as the ditrimethylsilyl ether, on a 6-ft. glass column packed with 5% SE-30 on Gas-Chrom Q (80–100 mesh). Column temperature, 240°; carrier gas, N_2 at 20 p.s.i. (1 μl = 20 μg). Peaks: C = chloramphenicol; IS = internal standard.

Fig. 2. Gas chromatogram of chloramphenicol as the trimethylsilyl ether on a 4-ft. glass column packed with 5% SE-30 on Gas-Chrom Q (80–100 mesh). Column temperature, 235°; carrier gas, N_2 at 15 p.s.i. (1μl = 20 μg). Peaks: C = chloramphenicol; IS = internal standard.

RESULTS

The retention times of chloramphenicol as the ditrimethylsilyl ether, with *m*-phenylene dibenzoate as internal standard, under different experimental conditions are shown in Figs. 1 and 2. Single determinations of a number of chloramphenicol samples were made by GLC, and the results were compared with the respective averages of several official assays. The peak areas were obtained with a disc integrator. The results, shown in Table I–III, reveal good correlation between the gas chromato-

TABLE I

GLC DETERMINATION OF CHLORAMPHENICOL WITH THE REAGENT OF BENTLEY *et al.*

Sample	*% of standard by GLC*[a]	*Official methods*	
		% by Lab. 1	*% by Lab. 2*
1	101.8	95.8	102.1
2	99.3	101.5	99.0
3	99.2	102.2	98.1
4	101.7	101.2	98.1
5	99.5	101.0	100.1
6	100.6	97.5	100.2
7[b]	100.1	—	101.6
8	100.6	99.4	101.5
9	101.1	100.5	100.5
10	100.7	—	—
11	100.2	—	—
12	97.8	99.0	100.2
13	102.2	98.5	101.0
14	100.0	99.4	100.0
15	100.0	100.0	98.7
16	100.2	103.5	100.0
17	100.2	99.3	101.8
18	101.4	99.1	100.5
19	99.1	97.8	102.6

[a] Samples 1–7: in pyridine, without use of internal standard, with 5% DC-200 column; samples 8–19: in acetonitrile, with *m*-phenylene dibenzoate as internal standard.
[b] Sample 7: capsule; all other samples: bulk.

TABLE II

GLC DETERMINATION OF CHLORAMPHENICOL BULK WITH BSA IN CH_3CN

Sample	*% Found*		*Official methods*	
	6-ft. column	*4-ft. column*	*% by Lab. 1*	*% by Lab. 2*
1	100.6	101.8	101.8	98.9
2	101.0	98.2	101.8	101.0
3	99.2	101.3	102.6	99.0
4	99.5	102.0	99.4	99.5
5	101.5	96.3	99.2	100.3
6	100.4	99.6	100.0	100.3
7	100.1	98.5	101.5	100.0
8	101.5	98.7	99.4	99.6
9	99.3	100.1	98.0	99.0
10	101.7	97.9	100.2	101.5
11	100.0	99.8	100.5	99.5

TABLE III

GLC DETERMINATION OF CHLORAMPHENICOL DOSAGE FORMS

Sample	*Form*	*% of label declaration*	*Official methods*	
			% by Lab. 1	*% by Lab. 2*
1	capsules	104.6	99.4	100.0
2	capsules	97.9	99.2	100.0
3	capsules	110.3	99.1	106.0
4	capsules	111.5	100.6	96.8
5	capsules	109.3	106.0	98.8
6	capsules	106.6	106.6	106.0
7	capsules	102.3	99.5	104.6
8	capsules	113.8	102.4	105.6
9	capsules	105.6	103	108
10	tablets	112.6	99.5	108.0
11	ophthalmic solution	113.9	126	119
12	injection	91.7	—	93.7
13	injection	97.1	—	93.7

graphic method and official assays. Replicate analyses of a solution from a capsule showed a precision of better than $\pm 3\%$.

DISCUSSION

The silylation method of BENTLEY *et al.*[11], employing hexamethyldisilazane and trimethylchlorosilane in pyridine, worked well when applied to chloramphenicol[7–9], as shown in Table I. However, after many analyses, difficulties arose as in the case of lincomycin[12], due mainly to contamination of the anode in the detector and the poor tailing characteristic of pyridine.

The use of a hydroxylic solvent such as methanol as a scavenger for the excess silylating reagent either directly or in extraction promoted solvolysis and inversion of configuration, particularly in pyridine or acetonitrile[10]. The silyl derivative prepared in pyridine, acetonitrile, ethyl acetate, acetone, dimethyl sulfoxide, and methylene chloride exhibited a single symmetrical chromatographic peak with quantitative recovery on an SE-30, DC-200, or QF-1 column. Although a change of solvent from pyridine improved the tailing character of the chromatogram, the siliceous deposits on the electrode remained the major concern. This was remedied by evaporating the reaction mixture to dryness and reconstituting in an inert solvent such as cyclohexane. That procedure, however, became too tedious for routine use.

As an alternative, the use of BSA[13] as a silylating reagent was investigated. The derivative obtained by the reaction of chloramphenicol with BSA showed the same retention time on three different columns as that of the O,O-ditrimethylsiloxy ether obtained with the reagent of BENTLEY *et al.* and previously confirmed by spectral techniques[10]. The BSA silylation procedure represents a marked improvement in several respects: (a) completion of the silylation reaction is indicated by complete dissolution and formation of a single-phase system (*i.e.*, no precipitate to clog the microsyringe); (b) the reaction is more rapid so that analysis can be achieved within minutes after derivatization begins; (c) the contamination of the electrode is materially reduced, resulting in greater longevity of the electrode and an overall increase in efficiency.

Specificity

The microbiological assay method is considered important primarily because the quantitation of antimicrobial activity is directly related to the end use of the product. That procedure is severely limited on a qualitative basis because numerous antibiotics may possess antimicrobial activity against a given test organism. Chemical tests are therefore recognized as necessary for the positive identification of antibiotics.

The GLC method clearly differentiates chloramphenicol from many structurally related compounds, several of which had been previously examined by four different methods[5]. It is noted that those compounds possessing the nitrophenyl group absorb near 275 mμ with an approximate molar absorptivity of 10,000 l/mole cm. As shown in Table IV, most of the chloramphenicol-related compounds chromatographed after silylation exhibited multiple peaks with much shorter retention times than that of chloramphenicol. The *meta* and *erythro* isomers of the drug (compounds IV and III) showed single symmetrical peaks which could be resolved from the one of interest. On the other hand, the chromatogram of the L-isomer (compound II) was identical to that of chloramphenicol and the chromatograms of compounds VIII, IX, and X were identical to each other, as were the chromatograms of compounds XIV, XV, and XVI. These findings are not unexpected, since it is known that enantiomers cannot normally be resolved on silicone columns. This shortcoming can be compensated by determining the specific optical rotation. The GLC method is specific for *threo-p*-nitrophenyl-2-dichloroacetamido-1,3-propanediol. It is believed that this GLC method may be improved to the point of resolving racemic mixtures by using a good high-temperature optically active liquid loading phase for the column.

TABLE IV

RELATIVE RETENTION TIMES OF CHLORAMPHENICOL AND SOME STRUCTURALLY RELATED COMPOUNDS AS SILYL ETHERS

No.	*Compound*	*Peaks*
I	Chloramphenicol	1.00[a]
II	L-*threo*-1-*p*-Nitrophenyl-2-dichloroacetamido-1,3-propanediol	1.00
III	DL-*erythro*-1-*p*-Nitrophenyl-2-dichloroacetamido-1,3-propanediol	1.1
IV	D-*threo*-1-*m*-Nitrophenyl-2-dichloroacetamido-1,3-propanediol	0.82
V	D-*threo*-1-*m*-Nitrophenyl-2-amino-1,3-propanediol	0.24 0.26 0.31
VI	D-*threo*-1-*p*-Nitrophenyl-2-amino-1,3-propanediol	0.28 0.31 0.33
VII	D-1-*p*-Nitrophenyl-2-acetamido-1,3-propanediol	0.53 0.55
VIII	D-1-*p*-Nitrophenyl-2-amino-1,3-propanediol	0.28 0.30 0.33 0.41
IX	L-1-*p*-Nitrophenyl-2-amino-1,3-propanediol	0.28 0.30 0.33 0.41
X	DL-1-*p*-Nitrophenyl-2-amino-1,3-propanediol	0.28 0.30 0.33 0.41
XI	DL-*threo*-1-Phenyl-2-dichloroacetamido-1,3-propanediol	0.28 (doublet)
XII	D-*threo*-1-Phenyl-2-formylamino-1,3-propanediol	0.13 0.15 0.18
XIII	DL-*threo*-1-Phenyl-2-nitro-1,3-propanediol	0.2[b]
XIV	D-*threo*-1-Phenyl-2-amino-1,3-propanediol	0.2[b]
XV	L-*threo*-1-Phenyl-2-amino-1,3-propanediol	0.2[b]
XVI	DL-*threo*-1-Phenyl-2-amino-1,3-propanediol	0.2[b]
XVII	*p*-Nitro-2-acetamino-β-hydroxypropiophenone	0.35[b] 0.41 0.51 0.59

[a] 9.51 min with the following instrument conditions: 6 ft. × 3 mm glass column packed with 5% DC-200 on Gas-Chrom Q (80–100 mesh); column temperature, 230°; FID; carrier gas, N_2 at 50 ml/min. Derivatives were prepared by the method of BENTLEY *et al.*

[b] Several additional peaks also noted with relative retention times of less than that listed.

Methodology

Column partition methods are being investigated for preliminary separation of chloramphenicol from interfering substances. These appear more promising and more efficient than liquid–liquid extractions. For sample preparation, ethyl acetate is preferred to methanol mainly because solutions of chloramphenicol in the former solvent appear to have greater stability over a two-week period. On the other hand, acetonitrile was selected as the reaction solvent because its use was associated with better precision and accuracy than ethyl acetate, although some inversion occurs upon standing, even after several hours. This is minimized by using a freshly prepared silylating reagent mixture just prior to chromatography.

Efforts will be made to extend the method of analysis just described to chloramphenicol esters such as the palmitate and the hemi-succinate.

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