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18. Magobei Yamamoto, Sadao Iguchi, and Toshinobu Aoyama : Determination of Chloramphenicol by Gas-Liquid Chromatography.

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Convenient gas-liquid chromatographic methods for the analysis of chloramphenicols have been described. A mixture of chloramphenicol and its acetates was completely separated and identified. For estimation, pharmaceuticals or plasma was extracted with appropriate solvent and chromatographed directly without extensive treatment utilizing hydrogen flame or electron capture detector system, respectively. The methods have speed, simplicity, and accuracy.

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The striking success of gas-liquid chromatographic procedure in the analysis of fatty acids, carbohydrates, and other biologically important materials suggested that this valuable technique might be applied with advantage to the determination of antibiotics and related compounds. It has already been shown that penicillin[1] and gibberellin[2] as their methyl esters, chloramphenicol[3] as its trimethylsilyl (TMSi) ether, and griseofulvin[4] without the need for further alternation can be successfully chromatographed. Recently, a quantitative measurement for chloramphenicol in serum has been described as the TMSi ether[5] which is an adaptation of a previously reported procedure.[3]

In this report, an investigation was conducted to develop the most convenient methods for the direct analysis of chloramphenicol with the purpose of pharmaceutical control and biochemical and/or biological studies.

Experimental

Apparatus and Materials——A Shimadzu Model GC-1B dual column gas chromatograph equipped with a Model HFD-1 dual hydrogen flame ionization system and with the two U-shaped stainless columns (75 cm. × 3 mm. i.d.) connected in series was used. Also employed was a Shimadzu Model GC-2C dual column gas chromatograph having a Model ECD-1A electron capture attachment (300 mc. Tritium Source) and a U-shaped borosilicate glass column (1.5 m. × 4 mm. i.d.). The glass column was constructed so that the sample was injected directly onto the heated chromatographic tube without the use of injection port. Nitrogen was used as the carrier gas for both instruments.

The column packings were (1) 1.5% SE-30 (methylsiloxane polymer, General Electric Co.), (2) 1.0% SE-52 (methylsiloxane phenylsiloxane copolymer, General Electric Co.), on Anakrom (90~100 mesh) (Anarabs Inc.), acid washed and treated with dimethyldichlorosilane, and (3) 2% DEGS (diethyleneglycol succinate polyester, Applied Science Laboratories, Inc.) on Anakrom (90~100 mesh), acid washed and coated with 1% PVP (polyvinylpyrrolidone K-25, Badische Anilin und Soda Fabrik) for inactivation of support.[6] The slurry coating technique of Horning, *et al.*[7] was used both for PVP coat (methanol solution) and the stationary phase coat (toluene solution for SE-30 and SE-52, acetone solution for DEGS). Prior to use, the packed columns were conditioned for 30 hr. at 260° for (3), 300° for (1) and (2), respectively.

Unless otherwise indicated, the gas chromatographic parameters were as follows : Model GC-1B—injection port temperature, 250°; column temperature, 195°; detector temperature, 210°; nitrogen flow rate, 20 ml./min. at inlet pressure of 2 kg./cm^2; sensitivity, 1000 MΩ; range, 1.6; hydrogen flow rate, 30 ml./min.;

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air flow rate, 1.0 L./min. Model GC-2C—injection port, column and detector temperature, 205°; nitrogen flow rate at outlet, 100 ml./min. for (3) and 140 m./min. for (1), (2); applied voltage, 10 V for (3) and 17 V for (1), (2); sensitivity, 100 MΩ; range, 1.6 for (3) and 6.4 for (1), (2).

Trimethylsilylation for Identification[8]——About 0.1 mg. each of chloramphenicol and its monoacetate were dissolved in 0.5 ml. of pyridine and treated with 0.01 ml. of each of trimethylchlorosilane and hexamethyldisilazane. After standing for 10 min. at room temperature, the mixture were diluted with pyridine as appropriate. Approximately 1 μl. of supernatant was introduced.

Calibration for Direct Determination——The internal standard technique utilizing HFD-1 for pharmaceuticals, and the amount increasing technigue utilizing ECD-1A for biological fluids were employed, respectively. The peak areas were determined by planimeter. Linear calibration curve in the former was obtained throughout the range of 10 to 30 mg. of pure chloramphenicol in each 1 ml. of 0.2% of dibutyl phthalate as internal standard in acetone, and also in the latter, the range of 0.1 to 1 μg. with isoamyl acetate solution.

Sample Preparation——A quantity of finely pulverized mixture of tablet equivalent to 23 to 73.1 mg. fortified with 6.2 to 19.8 mg. of pure chloramphenicol was extracted with 20 ml. of acetone by vigorous shaking for 2 min. The extract was filtered through the glass filter and the filtrated was evaporated to dryness. The residue was dissolved in accurate 4 ml. of 0.2% of dibutylphthalate in acetone. About 1 to 2 μl. of the solution was injected, the ratio of the peak areas again determined, and the amount of chloramphenicol was calculated from comparison with the calibration curve.

0.5 ml. of dog heparinized plasma containing chloramphenicol was buffered to pH 7[9] with 2 ml. of Clark Lubs solution, then, accurately 3 ml. of isoamyl acetate was added, and the mixture was shaken vigorously for 5 min. After brief centrifugation at 3000 r.p.m., accurately 1 μl. of aliquot of the supernatant was injected directly into the gas chromatograph without prior concentration. The peak area determined was compared to the calibration curve for calculation.

Result and Discussion

Since the conversion of a hydroxyl group to its TMSi ether or trifluoroacetate has the effect of increasing the volatility and decreasing the polarity of hydroxyl containing compounds, such derivatives were undoubtedly useful for gas chromatographic separation of relatively non-volatile *p*-nitrophenylserinol (D-*threo*-1-(*p*-nitrophenyl)-2-amino-1, 3-propanediol) derivatives. A mixture of chloramphenicol, its 3-acetate and 1,3-diacetate was separated by direct injection on SE-52 column but the resolution was unsatisfactory for routine analysis (Table I). Only by trimethylsilylation, the successful separation of the mixture was readily achieved on SE-30 phase giving the peaks in the elution order of chloramphenicol diTMSi ether, 3-acetate TMSi ether, and diacetate as illustrated in Fig. 1. The resolution might be used in biochemical separation problem such as enzymatic inactivation of chloramphenicol.[10]

Table I. Retention Time (t_R) for Chloramphenicol and Derivative

	Relative t_R (t_R)			
	1.5% SE-30[a] 205°	1.0% SE-52 205°[b]	1.0% SE-52 195°[c]	2.0% DEGS 195°
N-Acetyl-*p*-nitrophenylserinol			4.53	0.59
p-Nitrophenylserinol			5.18	0.59
Chloramphenical	1.00(5.1)	1.00(0.8)	1.00(0.85)	0.59
Dibutyl phthalate (I.S.)				1.00(3.7)
Chloramphenicol 3-acetate	1.39	14.1		
Chloramphenicol 1,3-diacetate	1.80	29.5		

a) AS TMSi ether. *b*) Without pretreatment both using ECD-1A.
c) Without pretreatment using HFD-1, respectively. For conditions see text.

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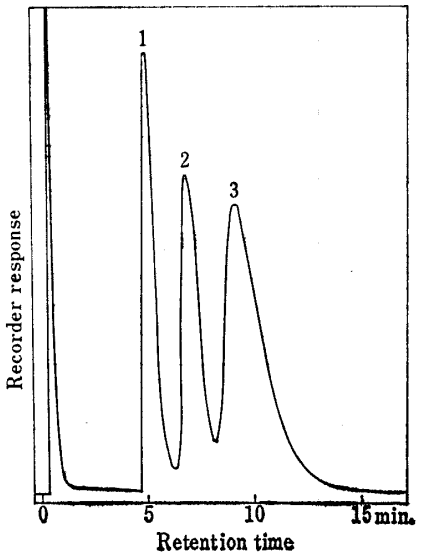

Fig. 1. Gas Chromatogram of a Mixture of Chloramphenicol and Its Acetates by Trimenthylsilylation

1: chloramphenicol diTMSi ether
2: chloramphenicol 3-acetate TMSi ether
3: chloramphenicol diacetate
1.5% SE-30, 205°, electron capture detection system

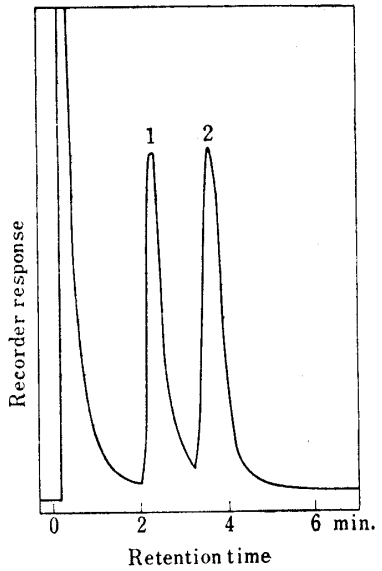

Fig. 2. Gas Chromatogram of Chloramphenicol and Dibutyl Phthalate

1: chloramphenicol
2: dibutyl phthalate (internal standard)
2% DEGS, 195° hydrogen flame ionization detection system

A gas chromatographic determination for chloramphenicol without derivation or extensive pretreatment was of interest to us because its high speed, simplicity and accuracy of the method would overcome the time-consuming and tedious manipulations of the others investigated. The stationary phase useful in this procedure was the polar DEGS in conjunction with PVP as tailing reducer. A typical chromatogram on DEGS phase is shown in Fig. 2 and retention data in Table I. Apparently, chloramphenicol, *p*-nitrophenylserinol, and N-acetyl-*p*-nitrophnylserinol had identical retention and therefore were detected as a single compound.

The SE-52 phase, however, gave the resolution of these three compounds with different responses of hydrogen flame detector.

To establish that chloramphenicol peak was not a thermal degradation product, the peak obtained by direct injection onto the heated glass column (180 to 220°) without the use of injector was compared to those with injector (190 to 250°) both utilizing the same DEGS packing and electron capture detector. This was verified by the complete identity for each peak with peak height and retention value, and also by the fact that both effluents corresponding to the peaks collected at the outlet of the column presented nearly the same ultraviolet spectra $\lambda_{max}^{H_2O}$ 276 mμ as the starting material $\lambda_{max}^{H_2O}$ 278 mμ (Fig. 3).

No interfering peak was found on analysing chloramphenicol on pharmaceutical preparations.

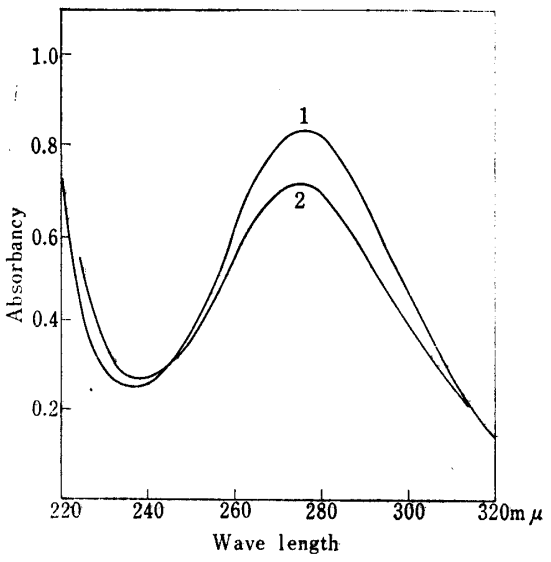

Fig. 3. Ultraviolet Spectra of Chloramphenicol before and after Chromatography

1: original, $\lambda_{max}^{H_2O}$ mμ: 278
2: effluent without injector $\lambda_{max}^{H_2O}$ mμ: 276

The recovery studies of chloramphenicol were carried out. Ten synthetic pulverized mixtures of commercially available tablets were analysed subjected to this proposed method, both before and after fortification with known quantities of pure chloramphenicol. The results shown in Table II illustrate quantitative recovery at different concentration. The proposed procedure gives average recovery of 99.2±3.0% well within the range of accuracy considered acceptable for pharmaceutical control.

Table II. Recovery of Chloramphenicol from Fortified Tablet

Initially present (mg.)	Added (mg.)	Total (mg.)	Found (mg.)	Recovery (%)
73.1	19.8	92.9	90.0	97.0
	20.2	93.3	88.6	95.0
50.0	28.0	78.0	80.2	102.9
	30.9	80.9	83.1	102.9
48.7	40.0	88.7	88.3	99.5
	43.8	92.5	90.4	97.7
32.3	49.5	81.8	78.0	95.4
	51.5	83.8	82.6	98.6
23.0	61.6	84.6	84.6	100.0
	62.0	85.0	87.8	103.2
			average	99.2
			st. dev. ±	3.0

Table III. Recovery of Chloramphenicol from Plasma

Added μg./ml.	Buffer		Plasma	
	Found μg./ml.	Recovery %	Found μg./ml.	Recovery %
20	18	90	16	80
	19	95	16	80
40	39	97.5	33	82.5
	40	100	32	80
60	63	105	50	83.4
	63	105	50	83.4
80	78	97.5	66	82.5
	78	97.5	88	85
100	98	98	86	86
	99	99	83	83
	average	98.4	average	82.6
	st. dev. ±	4.4	st. dev. ±	2.1

U-shaped stainless column (1.5 m. ×3 mm. i.d.) an injection port at 240° were used. For others see text.

Extention of this procedure into the nanogram range is required for separating and estimating chloramphenicol in biological fluids, and thus the use of electron capture detection system has advantage because the chloramphenicol molecule contains the high electron affinity groups. The results of the recovery experiment involving the extraction and direct injection of chloramphenicol added to dog heparinized plasma were presented in Table III. The average overall recovery of 10 to 50 μg. of chloramphenicol added to 0.5 ml. of plasma in the order of 82.6±2.1% was inconsistent with that of 98.4±4.4% obtained to buffer as blank. The almost constant difference of 15.8% is perhaps attributed to the bound chloramphenicol to plasma, and therefore, a correction factor will be necessary for estimating total chloramphenicol in plasma. The lower limit of sensitivity of the overall procedure was found to be 0.01 μg. This indicates that the

method should be potentially useful for rapid estimating free chloramphenicol in biological fluids.

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