THE CHARACTERIZATION AND THIN-LAYER CHROMATOGRAPHIC QUANTITATION OF THE HUMAN METABOLITE OF 7-DEOXY-7(S)CHLOROLINCOMYCIN (U-21,251 F)

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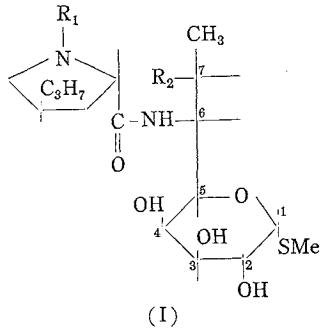
U-21,251 is 7-deoxy-7(S)-chlorolincomycin. The antimicrobial spectrum of this new antibiotic is extended and it is about twice as active as lincomycin *in vivo* against gram-positive infections. During routine analysis of biological fluids obtained from test subjects, a bio-activity chromatographically different from the 7-deoxy-7(S)-chlorolincomycin was observed on thin-layer bioautograms. This metabolite has not been differentiated from the N-demethyl-7-deoxy-7(S)-chlorolincomycin by thin-layer chromatography (TLC). Although we have not recovered sufficient metabolite from urine for unequivocal identification, some indirect evidence supporting the structural assignment is presented. A TLC quantitative analysis for the 7-deoxy-7(S)-chlorolincomycin and its metabolite has been developed for biological fluids, particularly serum. The analysis is based on the separation of the two activities on silica gel and subsequent quantitation by regression analysis of the zone sizes obtained by bioautography. The regression lines cover the range 0.5 to 0.0038 mcg and show a maximum variation of 3.0 %, based on intercepts. This variation covers contributions by serum and TLC plates. Samples, which usually required concentration prior to analysis, may be quantitated with a 7.5 % standard error.

In 1962 MASON *et al.*[3,5] described the discovery and isolation of the antibiotic, lincomycin (I). It is a novel antibiotic structurally[2,6,7] in that an octopyranoside containing both nitrogen and sulfur is linked through an amide bond to 1-methyl-4-*n*-propyl-L-proline.



(I)

	R_1	R_2
Lincomycin	CH_3	OH(R)
7-Deoxy-7(S)-chlorolincomycin	CH_3	Cl(S)
N-Demethyl-7-deoxy-7(S)-chlorolincomycin	H	Cl(S)
N-Demethyl lincomycin	H	OH(R)

In one of the chemical modifications of lincomycin, the hydroxyl group at position seven is replaced with chlorine[4]. The resulting 7-deoxy-7(S)-chlorolincomycin

demonstrated *in vitro* antibacterial activity approximately 4~8 times that of lincomycin and about twice the oral *in vivo* activity when administered to infected mice. As part of the investigation of 7-deoxy-7(S)-chlorolincomycin, samples of biological fluids from human test subjects were assayed by the usual disc-plate or cup-plate techniques. In addition, selected samples were bioautographed after chromatography on silica gel thin-layer plates to characterize the biological activity. As a result of this chromatography, a second biological activity, in addition to 7-deoxy-7(S)-chlorolincomycin was found in the serum, urine and fecal samples. This unidentified component was subsequently differentiated by chromatographic techniques from all suspected lincomycin analogs except N-demethyl-7-deoxy-7(S)-chlorolincomycin[4]. Attempts were made to isolate sufficient material from urine to accomplish complete physical-chemical characterization. Although it was not possible to isolate the pure compound in sufficient quantity to rigorously establish its identity, ample evidence was obtained to conclude that the metabolite is N-demethyl-7-deoxy-7(S)-chlorolincomycin.

To augment *in vivo* studies, it was necessary to establish a method for quantitating the parent and metabolite in biological fluids, particularly serum. The dose response of both antibiotics in fresh human serum was determined and based on these results, a differential thin-layer chromatographic (TLC) analysis was developed. The standard regression lines for these antibiotics cover three orders of magnitude (0.52~0.0038 mcg) and show a maximum variation among subjects of 3.0% based on their intercepts. Human serum samples were quantitated with a standard error of 7.5%, at levels between 0.03 and 2.1 mcg/ml.

Experimental

Characterization of the Metabolite

All samples were chromatographed on silica gel H thin-layer plates with Mobile phase A of the following composition: methyl ethyl ketone 186 parts, acetone 52 parts, water 20 parts.

Sample Preparation for Chromatography

Sera were denatured with equal volumes of acetone and centrifuged. The supernatant was then collected for chromatography. In some cases, urine samples were applied directly while in other experiments they were treated with acetone as described above. Some urine samples were adjusted to pH 10 with sodium hydroxide and subsequently extracted with *n*-butyl alcohol. The extracts were reduced to dryness under vacuum and the residues dissolved in a minimum of acetone. Reduction of the acetone volume resulted in a solid which was used for characterization.

Purification of the Metabolite

Forty gallons (151.1 liters) of urine from test subjects receiving 7-chloro-7(S)-chlorolincomycin was adjusted to pH 10 and extracted with an equal volume of *n*-butyl alcohol. The exract was reduced to dryness and the residue distributed in a solvent system consisting of 1/1 : *n*-butyl alcohol/water in a CRAIG counter double current distribution device. The samples were introduced through the center tube and after 100 transfers, fractions were collected and analyzed by bioactivity, solids analysis and TLC. The fractions which contained the suspected metabolite were concentrated to dryness and further purified by preparative TLC. Methanolic solutions containing up to 300 mg of solids of the various

CRAIG fractions were applied to 2 mm thick silica gel G TLC plates and irrigated in system A. The components representing biologically active material were located by applying 1/2 inch Whatman No. 1 paper strips saturated with water to the surface of the silica gel matrix. After ten minutes the strips were removed and bioautographed on *Sarcina lutea*. The Rf's of the resulting bioautographic zones were correlated with the TLC matrix and the silica gel in these regions was removed with a device previously described[1]. These silica gel samples were then extracted with methanol, the methanolic extracts reduced to dryness, and the resulting solids used for infrared and mass spectrometric analysis.

Infrared Spectrometry

All infrared spectra were run on a Perkin-Elmer Model 137B using sodium chloride optics. The frustrated multiple internal reflectance spectra were obtained with a Wilks (Wilks Scientific Corporation, Norwalk, Connecticut) Model 2A FMIR optical system using a 2×51 mm KRS5 plate cut at 45°C.

Quantitation of Serum Components

Dose Response

The dose response and TLC resolution of 7-deoxy-7(S)-chlorolincomycin and its N-demethyl analog were obtained in fresh human serum on silica gel H TLC plates. Whole blood was collected from three subjects and allowed to clot overnight at 40°C. After high speed centrifugation the serum was collected and used to prepare solutions containing 100 mcg/ml each of the two antibiotics. These solutions were then serially diluted with each subject's serum to 6.25 mcg/ml. An equal volume of acetone was added to each sample and after agitation, they were centrifuged and the supernatant collected. Ten mcl of the resulting acetonates, containing from 50 to 3.125 mcg/ml of each antibiotic, were applied to silica gel H (0.3 mm) plates and irrigated in mobile phase A. After drying, the plates were bioautographed on *Sarcina lutea* as described in a previous publication[1]. The regression lines were extended to 0.0031 mcg by applying 10, 5, 2 and 1 mcl aliquots of the 3.125 mcg/ml dilution to silica gel H plates and chromatographing them as described.

Analytical Procedures

Using selected subject's zero hour sera, standard solutions containing from 100 to 0.75 mcg/ml of each antibiotic were prepared. The solutions were treated with acetone as described previously and supernatants collected. To prevent overlapping of the zones on the bioautograms, the acetonates were applied at 10 mcl each on three silica gel H thin-layer plates in the following sequence; 0.5, 0.0625, and 0.03125 mcg; 0.25, 0.015, and 0.0075 mcg; 0.12, and 0.0038 mcg. The plates were irrigated as described and subsequently bioautographed on *Sarcina lutea*. The zones were measured to the nearest millimeter and the values used with the log of doses to obtain regression lines on an IBM 360 computer.

The serum samples used for quantitation were treated in the same manner as described for the standard solution except that 4 volumes of acetone was used for denaturation. The supernatant acetonates were then stripped of acetone under vacuum and the resulting aqueous solutions lyophilized. The lyophilates were taken up in 0.1 the volumes of the original serum samples in 50% aqueous ethanol. Replicate samples were spotted on either the same silica gel H thin-layer plate or on different plates. Plates were irrigated in mobile phase A and bioautographed as described. The zone sizes and sample volumes were used in the regression program to obtain the quantity of antibiotic in the serum.

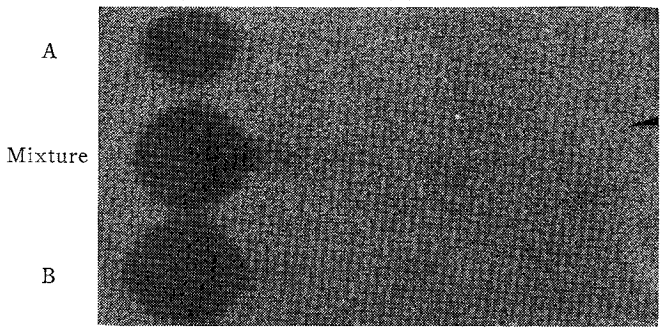
Results and Discussion

Characterization of the Metabolite

A bioautogram of a mixed and parallel thin-layer chromatogram of subject urine and authentic N-demethyl-7-deoxy-7(S)-chlorolincomycin is shown in Plate 1. These

zones are easily distinguished from most other bioactive lincomycin derivatives and analogs based on their Rf values.

Plate 1. Mixed and parallel TLC bioautogram of subject urine (A) and N-demethyl-7-deoxy-7(S)-chlorolincomycin (B).



Although extraction of test subjects' urine with *n*-butyl alcohol removed the biological activity nearly quantitatively, contamination of the extracts with urea and other urine solids precluded spectral interpretation. The extracts were, therefore, further purified by CRAIG double cuntercurrent distribution. The FMIR spectrum of preparative TLC purified CRAIG solids which corresponded chromatographically (TLC) to N-demethyl-7-deoxy-7(S)-chlorolincomycin is shown in Fig. 1. Although the spectrum of the CRAIG solids lacks some definition, it was sufficiently characteristic of lincomycin analogs to warrant its examination by mass spectrometry. The mass spectrum in

Fig. 1. FMIR spectra of CDCD extract of subject urine (A) and 7-deoxy-7(S)-chlorolincomycin (B).

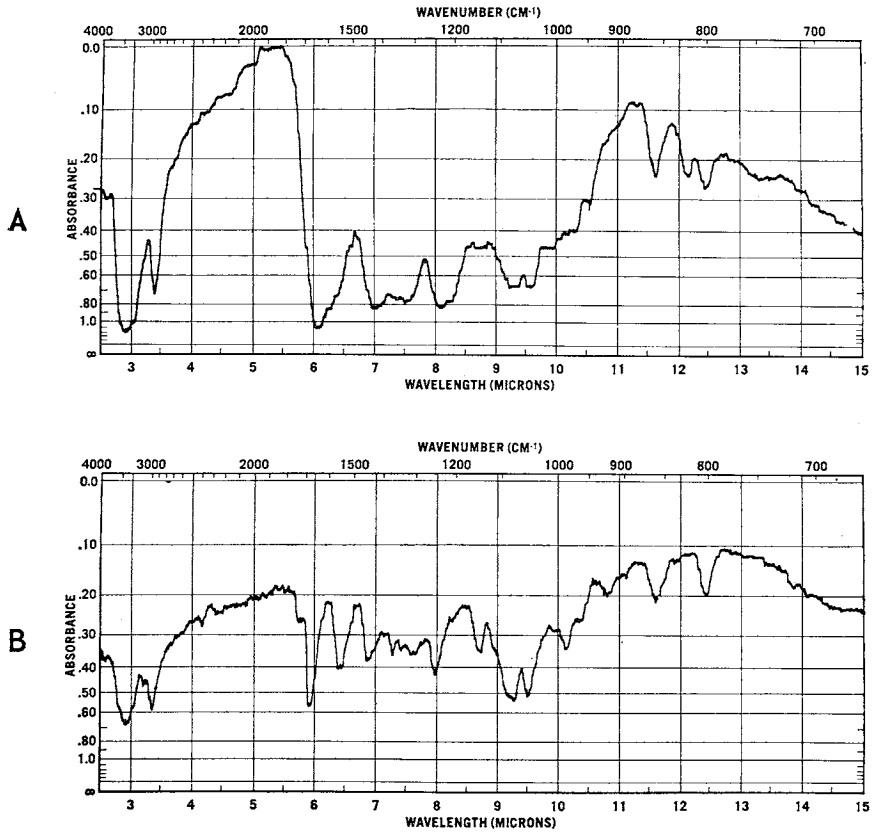
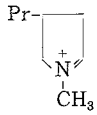
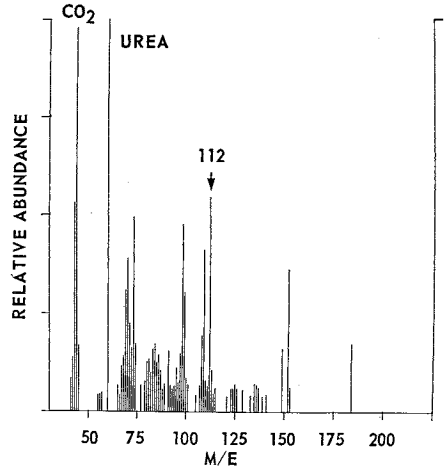


Fig. 2 lends support to the chromatographic evidence that the human metabolite of 7-deoxy-7(S)-chlorolincomycin is the corresponding N-demethyl analog. Disregarding the 66 (urea) and 44 (CO_2) m/e peaks, then 112 m/e is the base peak. There is only a minor 126 m/e peak observable in this spectrum. It is known that lincomycin and 7-deoxy-7(S)-chlorolincomycin give 126 as the base peak which results from the fragment II, and the latter antibiotic has been identified in CRAIG fractions corresponding to 7-deoxy-7(S)-chlorolincomycin by TLC:

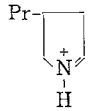


(II)

Fig. 2. Mass spectra of TLC purified CDCD fractions of subject urine.



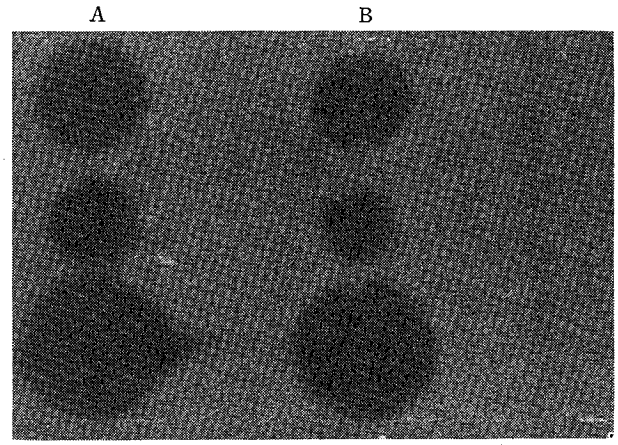
Further, N-demethyllincomycin (I) gives a 112 m/e base peak resulting from fragment III, therefore, by analogy, the 112 m/e in the CRAIG fractions corresponding to N-demethyl-7-deoxy-7(S)-chlorolincomycin by TLC is assigned to fragment III.



(III)

The 126 m/e peak in the latter spectrum might be accounted for by trace contamination with 7-deoxy-7(S)-chlorolincomycin.

Plate 2. TLC bioautograms of standards of 7-deoxy-7(S)-chlorolincomycin (B) and N-demethyl-7-deoxy-7(S)-chlorolincomycin (A).



Quantitation of Serum Components

A typical bioautogram of a portion of the standard curve for one of the original test subjects appears in Plate 2. The antibiotics were well separated and there was no difficulty with cross contamination. The precision with which a single individual's standard curve was reproduced is shown in Table 1. These values were used to indicate a probable assay model. In this case a linear log dose-response model was used.

Table 1. Reproducibility of regression lines for a single subject

Antibiotic	Ave. slope	% Std. Error	Ave. Intercept	% Std. Error
7-Deoxy-7(S)-chlorolincomycin	15.5	1.86	36.4	0.99
N-Demethyl analog	13.8	3.02	37.7	1.95

Since the same samples were used for each determination, the values represent

Table 2. Variation of regression lines for two groups of subjects (based on Intercept)[a]

	7-Deoxy-7(S)-chlorolincomycin			N-Demethyl analog		
	Group 1	Group 2	Grand Ave.	Group 1	Group 2	Grand Ave.
Intercept #1	35.9	36.9		36.2	38.6	
Intercept #2	34.7	36.3		37.2	38.2	
Intercept #3	38.4	36.5		37.9	83.1	
Ave.	36.3	36.6	36.5	37.1	38.3	37.7
Std. Dev.	1.88	0.31	1.35[b]	0.85	0.27	0.63[b]
% Std. Error	3.0	0.49	1.75	1.33	0.41	0.87

a) The intercept was chosen since it is a reflection of the antibacterial zonal response for the antibiotics.

b) Average estimated Std. dev. $\bar{\delta}=\sqrt{\frac{\sum S^2}{n}}$

the day to day variation experienced with bioautographic trays and application errors. In order to determine the extent of variation resulting from the use of different subject zero hours sera, the intercepts of the various regression lines for two groups of subjects were averaged and a standard error computed, this is shown in Table 2.

The average standard errors are not significantly larger than those enountered for a single subject. Assuming that the zone sizes could not be read to better than 7.5 % (this is the standard error for all assays in this study), then the maximum 3.0 % variation would not preclude the use of pooled human serum in place of subject zero hours serum for making the serial dilutions. The higher average intercept for the N-demethyl analog is consistent with *in vivo* studies showing that the N-demethyl analog is about 4 times the activity of 7-deoxy-7(S)-chlorolincomycin.

For evaluation of the quantitation of the two antibiotics, subject sera was selected from a group of *in vivo* samples for workup according to the procedures described in the experimental section. The use of concentrated alcoholates in lieu of dilute acetonates allowed application of the solutions at levels (10~20 mcl depending upon the antibiotic level) more closely related to the level of the antibiotic standard. The limits of reproducibility of the slopes and intercepts for all subjects selected for this study are given in Table 3. The results of quantitation of two samples of subject sera is given in Table 4.

For these quantitations, the mean standard error of the average was 7.5 % for both antibiotics combined. Individually they were 7.3 % for 7-deoxy-7(S)-chlorolincomycin and 7.7 % for its N-demethyl analog. These values were

Table 3. Statistics for the reproducibility of regression lines for all subjects for all runs*

	7-Deoxy-7(S)-chlorolincomycin	N-Demethyl analog
Ave. Slope	14.8	13.3
Std. Dev.	0.93	1.1
% Std. Dev.	6.3	8.1
UCL (95 %)	15.4	13.9
LCL (95 %)	14.2	12.6
Ave. Intercept	36.5	37.9
Std. Dev.	0.88	0.72
% Std. Dev.	2.4	1.9
UCL (95 %)	37.0	38.3
LCL (95 %)	35.9	37.4

* These values represent 12 runs.

Table 4. Specific serum quantitations for 2 subjects

Subject	Antibiotic	Level (mcg/ml) 1~1/2 hr (Avg.)	Level (mcg/ml) 8 hr (Avg.)
1	N-Demethyl analog	0.03	0.04
	7-Deoxy-7(S)-chlorolincomycin	0.89	0.13
2	N-Demethyl analog	0.04	0.03
	7-Deoxy-7(S)-chlorolincomycin	2.1	0.28

calculated on the bases of three replicate determinations for each of 15 quantitation experiments. The values in Table 4 represent typical limits of antibiotic levels encountered.

During this investigation it was observed that the response of both antibiotics varied markedly as a function of the age of the TLC plates used. The nature of this phenomena is not known but this observation does make it imperative that all quantitations be performed on plates prepared in the same batch.

Acknowledgements

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