Die Frkt. b. wurde durch Sublimieren im Vakuum (0.002 mm Hg-Druck) in weitere 3 Teile getrennt.

Frkt. b_1 120~150°(Badetemp.)	spur	amorph
Frkt. b_2 150°(Badetemp.)	1 mg	gelbes Blättchen
Frkt. b_3 150~180°(Badetemp.)	2 mg	kristallinisch

Die Frkt. b_3 wurde nochmals im Vakuum (0.002 mm Hg-Druck) sublimiert und in 3 Teile getrennt.

Frkt. $b_{3.1}$ 120~150°(Badetemp.)	spur	amorph
Frkt. $b_{3.2}$ 150°(Badetemp.)	1 mg	prismatische Nadeln
Frkt. $b_{3.3}$ 150~200°(Badetemp.)	1 mg	prismatische Nadeln

Die Frkt. b_2, $b_{3.2}$ und $b_{3.3}$ wurden in das Pikrat übergeführt und je aus Chloroform umkristallisiert. Die Pikrate aus $b_{3.2}$ und $b_{3.3}$ sind schwer zu reinigen und zeigten den Schmp. 252~260°. Das Pikrat aus Frkt. b_2 (Nr. 8) bildete Prismen vom Schmp. 269~273°, welche bei einer Mischprobe mit dem Pikrat vom nach Mosettig[7] hergestellten 1-Azachrysen (Schuppen, Schmp. 276~279°) bei 273° schmolzen. Die UV-Spektren der freien Base stimmten mit denen des 1-Azachrysen fast überein, wie in der Tabelle II vergleichend zusammengestellt wurden.

Tabelle II.

Synthetisches Präparat				aus Pikrat Nr. 8	
Johnson, *et al.*[5]		Hara			
λ_{max}	log ε	λ_{max}	log ε	λ_{max}	log ε
263	4.9	266	4.8	365	4.8
298	4.3	299	4.35	300	4.2
359	3.35	361	3.45	361	3.4
λ_{min}	log ε	λ_{min}	log ε	λ_{min}	log ε
230	4.3	230	4.25	233	4.35
293	4.1	294	4.1	294	4.1
353	3.0	355	3.0	355	3.15

Zusammenfassung

Die Konstitutionsermittelung der vier Lactame A, B, C und D, die in der vorliegenden Mitteilung beschrieben sind, wurde ausgeführt. Hiermit wurde das Lactam-C als das 4-Aza-A-homocholanderivat und das Lactam-A als das 4-Azacholanderivat festgestellt. Infolgedessen stellt das Lactam-B bzw. -D je das entsprechende 3-Azaderivat dar.

(Eingegangen am 19. Mai, 1955)

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61. **Takeichi Sakaguchi and Kiyomi Taguchi**: Colorimetric Determination of Aureomycin in Biological Materials by Butanol-Thorium Extraction Method.

(*Pharmaceutical Faculty, University of Chiba**)

In earlier paper by the present authors,[1,2] a sensitive colorimetric determination of Aureomycin based on chelate formation with thorium(IV) was described.

This colorimetric method, however, is subject, in varying degrees, to interfering substances normally present in biological fluids. Consequently, the antibiotic must be separated from biological materials by extraction or absorption,[3] prior to its colorimetric determination. The method described here effects the extraction, based upon the solubility of Aureomycin Hydrochloride in butanol, of its thorium chelate in water, and on

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1) M. Ishidate, T. Sakaguchi: This Bulletin, **3**, 145 (1955).
2) T. Sakaguchi, T. Taguchi: *Ibid.*, **3**, 166 (1955).
3) Saltzman: J. Lab. Clin. Med., **35**, 123 (1950).

the insolubility of the chelate in butanol. In most biological systems such as body fluids Aureomycin exists presumably in the form of metal chelates. The following experiments indicate that sodium ethylenediaminetetraacetate (EDTA) is able to mask these metals which are in complex combination with Aureomycin and the effective extraction of Aureomycin from body fluids is possible.

The Aureomycin taken up in butanol was transferred to the aqueous medium through the thorium reagent and was determined colorimetrically in the form of an Aureomycin-thorium chelate.

Achromycin and Terramycin were also determined by the same method. The zirconium chelate evidenced poorer properties when compared with thorium chelate because of its lower absorbability or liability to precipitate. It has been found difficult to obtain reproducible extraction using chelating metals other than thorium, due to the inherent instability of most metal-Aureomycin chelates in acidic solution and to the less sensitive intensity of the color produced.

Experimental

Reagents—$Th(NO_3)_4$ solution, 1%: Prepared by dissolving 1 g. of the reagent grade $Th(NO_3)_4 \cdot 4H_2O$ in 100 cc. of distilled water and adding 1 drop of conc. HNO_3.

Na ethylenediaminetetraacetate solution, 10%: Prepared by dissolving 10 g. of Na_2 ethylenediaminetetraacetate dihydrate in 90 cc. of distilled water.

AcONa·AcOH buffer solution, 5*M*: Prepared by dissolving 30.0 g. of pure glacial AcOH and 68.0 g. of pure $AcONa \cdot 3H_2O$ in freshly distilled water and diluting to 200 cc. The pH of this solution is about 4.6.

HCl, 15%: A solution consisting of 7 volumes of water and 3 volumes of conc. HCl.

NaCl Solution, 25%: 50 g. of NaCl dissolved in 150 cc. of distilled water.

BuOH: c.p. grade.

NaCl: c.p. grade.

Apparatus—A Hitachi Photoelectric Spectrophotometer, Model EPU-2, was used for all absorption measurements using 1-cm. cells. Cell corrections were made when necessary.

Procedure—Sample preparation: A solution containing approximately 2~20 γ/cc. of Aureomycin Hydrochloride was prepared. In the case of blood samples 2 cc. of 10% ethylenediaminetetraacetate solution was added to each 5 cc. of the sample. EDTA solution acts as an anticoagulant by combining with Ca ions in the blood. 5~10 cc. aliquot of the sample solution was pipetted into a stoppered centrifuge tube and 1~2 cc. of 10% EDTA solution was added. The pH was adjusted to 1~2 with 15% HCl solution. A sufficient amount of NaCl to saturate the solution was added to the above tube for salting out, and it was balanced against a control tube, containing the same amount reagents, by the addition of NaCl.

Extraction with BuOH: 4 cc. of BuOH was added to 10 cc. of the sample solution saturated with NaCl. The mixture was shaken vigorously for about 3 mins., allowed to settle, and centrifuged for 5~15 mins. at 3000 r.p.m. The upper layer was transferred with a capillary pipet into another stoppered centrifuge tube. The extraction was repeated 3~4 times with about 2 cc. each of butanol.

Washing of BuOH layer: 2 cc. of 25% NaCl solution was added into the second centrifuge tube in which the BuOH extract had been collected, and the mixture shaken vigorously. After centrifuging, the lower layer was discarded. The washing with 25% NaCl solution was repeated once more.

Extraction with 1% $Th(NO_3)_4$ solution: 2 cc. of 10% $Th(NO_3)_4$ solution was added to the washed BuOH layer, the mixture was shaken vigorously for about 2 mins., and centrifuged. The lower layer containing the Aureomycin-Th complex was separated with a capillary pipet into a 10-cc. measuring cylinder. Extraction was repeated with 3 portions of 2 cc. each of $Th(NO_3)_4$ solution.

For the analysis of samples containing less than 20 γ (2 γ/cc.) of Aureomycin Hydrochloride, it is necessary to have as little volume of $Th(NO_3)_4$ solution as possible as increase of the volume of thorium extract lessens the measurable range of Aureomycin. The extraction was carried out first with 2 cc. and then with 3 portions of 1 cc. of Th reagent. The combined extract was made up exactly to 5 cc. with distilled water and followed by the addition of 0.5 cc. of 5 *M* AcOH-AcONa buffer solution.

Preparation of samples for photometric determination: One cc. of 5 *M* AcONa-AcOH buffer solution was pipetted into a meauring flask, made up to 10 cc. with distilled water, and thoroughly

mixed. The pH of this solution should be 4.0~4.2. Filtration was carried out, if necessary. The absorbance was determined 20 mins. later at 400 mμ against a blank of distilled water. The calibration curve was prepared by making 10cc. of several known concentrations of Aureomycin Hydrochloride and analyzed as outlined above.

In Tables I and II, the results are illustrated on the extracted samples and standard solution (Fig. 1).

Table I. Extracted Samples.

AM-HCl (γ/cc.)	2	5	10	15	20
Absorbance at 400 mμ	0.085	0.195	0.372	0.571	0.750
〃	0.087	0.188	0.365	0.590	0.750
〃	0.087	0.190	0.390	0.565	0.765
〃	0.088	0.190	0.376	0.574	0.756
Mean Value	0.087	0.191	0.376	0.574	0.756

Table II. Standard Solution

AM-HCl (γ/cc.)	2	5	10	15	20
Absorbance at 400 mμ	0.090	0.210	0.385	0.580	0.780
〃	0.088	0.200	0.400	0.590	0.780

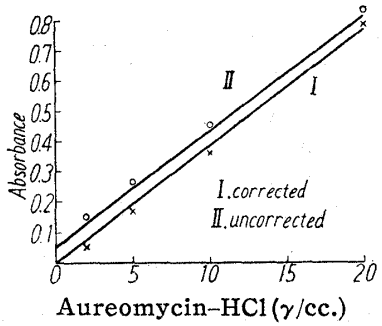

Fig. 1.

The concentration of Aureomycin Hydrochloride by the extraction may be calculated by the following relationship :

$$C = 26.5 \times A_{400}$$

where C is the concentration of Aureomycin-HCl (γ/cc.) in the sample, A_{400} is the absorbance of the sample at 400 mμ using 1-cm. cells, and 26.5 is a coefficient calculated from molar absorptivity of Aureomycin-Th complex at 400 mμ ($\varepsilon = 19,400$).

Determination in Urine : In the analysis of urine samples by Th-BuOH extraction, it was found that the absorbance of interfering substances considerably increased in proportion to the decreasing amount of Aureomycin Hydrochloride, at less than 20 γ/cc. The absorbance curve from interfering substances contained in urine samples was found to be a straight line in the wave length range of 420 to 380 mμ. The corrected absorbance of Aureomycin Hydrochloride was therefore calculated from the observed absorbances at three selected wave lengths by the equation of McGillivary[4] :

$$\text{Corr. } A_{400} = 1.75(2A_{400} - A_{420} - A_{380})$$

where Corr. A_{400} is the corrected absorbance at 400 mμ, A_{400}, A_{380}, and A_{420} are absorbances of the sample at 400, 380, and 420 mμ, respectively.

In Table III, the results are illustrated on urine samples containing known amounts of Aureomycin Hydrochloride.

Table III. Extracted Samples from Urine

AM-HCl (γ/cc.)	2		5		10		20	
Obs. A_{380}	0.125	0.148	0.200	0.200	0.340	0.335	0.538	0.575
Obs. A_{400}	0.130	0.156	0.280	0.237	0.425	0.412	0.810	0.835
Obs. A_{420}	0.091	0.120	0.165	0.171	0.303	0.282	0.515	0.544
Corr. A_{400}	0.078	0.078	0.166	0.180	0.362	0.359	0.774	0.787
Standard A_{400}	0.087		0.191		0.376		0.756	
Recovery of Corr. A_{400} %	90	90	87	94	97	95	102	103
Recovery of Obs. A_{400} %	150	179	120	124	113	110	107	110

4) W. A. McGillivary : Anal. Chem., **22**, 494(1950).

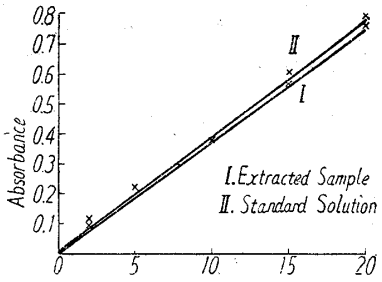

Fig. 2.

Number of BuOH extractions : Extraction of 10 cc. of the test solution with 2-cc. portions of BuOH should be repeated a total of three times as is obvious from Table IV. The test solution was always saturated with NaCl and then adjusted to pH 2 with dil. HCl.

Table IV.

No. of extractions	1	2	3	4	5	6
Absorbance	0.82	0.89	0.94	0.93	0.94	0.93
Recovery %	88.3	95.7	101	100	101	100

The removal of water-soluble fraction from BuOH solution by shaking repeatedly with distilled water showed significant loss of Aureomycin (Table V).

Table V.

No. of washings	0	2	4	7
Absorbance	0.605	0.505	0.383	0.299
Loss %	0	16.5	36.7	50.6

Consequently, 25% NaCl solution should be used for the washing of the organic layer in the procedure mentioned above.

Number of extraction with 1% $Th(NO_3)_4$ solution : The extraction with 1% $Th(NO_3)_4$ solution was carried out as previously described and gave fairly consistent and reproducible results (Table VI). In the series of experiments illustrated in Table VI, 6 cc. of BuOH containing Aureomycin was always extracted with 1 cc. of $Th(NO_3)_4$ solution.

Table VI.

No. of extractions	2	3	4	5	6
Absorbance	0.900	0.950	0.960	0.955	0.955
Recovery %	94.0	99.5	100.4	100	100

Effect of buffer solution : The absorbance of Aureomycin-Th complex remained constant at 400 mμ within the pH range of 4.0~4.4. It was found desirable to keep the pH of Th reagent at a rather acidic value (about pH 2) to prevent its complex formation with interfering substances in the urine. Therefore, the pH of AcONa-AcOH buffer solution must be on the alkaline side to adjust the Th extract to the optimum pH. As seen from the results in Table VII, an adequate volume ratio of 5 *M* AcONa-AcOH buffer solution was 1 cc. to 8 cc. of 1% $Th(NO_3)_4$ solution (about pH 2).

Table VII.

5 *M* AcONa-AcOH buffer solution*(cc.)	A_{380}	A_{400}	A_{420}
0	0.277	0.300	0.145
0.1	0.286	0.302	0.143
0.5	0.260	0.340	0.245
0.8	0.250	0,340	0.251
1.0	0.250	0.340	0.255
1.4	0.250	0.340	0.255
2.0	0.242	0.315	0.236

* Amount of buffer solution to be added to 8 cc. of 1% $Th(NO_3)_4$ solution (about pH 2) containing Aureomycin.

Extraction and Colorimetric Determination of Aureomycin from Human Urine : Aureomycin Hydrochloride excreted into urine after its oral administration of 500 mg. (9 mg./kg.) was analyzed by this extraction method. The results are illustrated in Table VIII and Fig. 3.

Table VIII.

Time after administration (hrs.)	1	2	3	4	5	6	24
Volume of urine (cc.)	90	140	100	50	70	65	115
Aureomycin-HCl (γ/cc.)	0	16.6	70.2	120.3	100	85.0	4.5
Total AM-HCl (γ)	0	2324	7000	6015	7000	5525	517.5

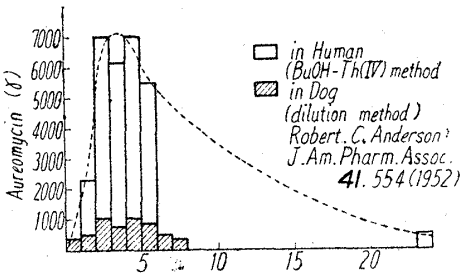

Fig. 3.

Determination in Blood—When the above extraction procedure was applied to blood samples, difficulty was encountered in complete separation of the proteins from the BuOH layer and was attributed to the high concentration of protein in blood. The problem was solved by heating the solution to 70° at pH 1.2 after the addition of a suitable amount of EDTA and BuOH to the sample.

Procedure—Preparation of the sample : Aureomycin in blood samples having a concentration of 2~20 γ/cc. was determined successfully by a method similar to that previously mentioned. A 10-cc. sample to which 4 cc. of 10% EDTA had been previously added to prevent coagulation, was placed in a stoppered centrifuge tube, 20 cc. of distilled water was added to this solution and, after adjusting to pH 1~2 with 15% HCl, 5 cc. of BuOH was added and heated to 70° on a water bath for about 3 mins. Finally, the content was saturated with solid NaCl and mixed vigorously for at least 3 mins. Separation was carried out by centrifugation, the supernatant liquid (BuOH) was transferred to another centrifuge tube either by decantation or pipetting. Three successive extractions were carried out without heating by shaking with additional portions of 5 cc. of BuOH as above.

Washing of BuOH layer : The combined BuOH extracts was washed twice with 2-cc. portions of 25% NaCl solution.

Extraction with 1% $Th(NO_3)_4$: This procedure is the same as previously described, except that the extracted Th layer is first transferred to a centrifuge tube instead of a measuring cylinder. The extraction of Aureomycin from the BuOH layer was carried out first with 3-cc. and then with three 2-cc. portions of 1% $Th(NO_3)_4$ solution. $Th(NO_3)_4$ solution (about 8 cc.) collected in a centrifuge tube was washed by shaking for a few minutes with 5-cc. portions of BuOH, until all traces of coloring substances were removed. After centrifugation, the supernatant liquid was taken off and the washed $Th(NO_3)_4$ solution was transferred to a measuring cylinder with a capillary pipet, followed by the addition of 1 cc. of water with which the content of the centrifuge tube was washed. One cc. of 5 *M* AcONa-AcOH buffer solution was then added, the final volume was made up exactly to 10 cc., and allowed to stand for 20 mins. Absorbance measurements were made at 400 mμ, using distilled water as a blank. The Aureomycin content of the sample was then determined from a standard curve (Fig. 1) or the calibration equation. Table IX shows results obtained on samples which contain different amounts of Aureomycin Hydrochloride. The calculated values were in close agreement with the observed ones obtained in this investigation.

Table IX. Blood Samples

AM-HCl added γ/cc.	Absorbace at 400 mμ	AM-HCl found γ/cc.
2.5*	0.109	2.6
2.5*	0.100	2.4
5.0	0.206	5.2
7.5	0.286	7.7
7.5	0.288	7.8
10.0	0.373	10.3

* Serum was used as the sample, whereby washing procedures were eliminated.

Discussion of Results

1. In the determination of Aureomycin in blood samples, it was found that protein interferes, as it combines either adsorptively or chemically with Aureomycin, forming complexes of Aureomycin with metals or proteins, or both. EDTA was found to be suit-

able for separation of Aureomycin from the protein complexes.

2. The coagulation of blood protein in the extraction procedures has variable physical characteristics, particularly in regard to viscosity, and causes the separation by butanol to be difficult. This problem, which becomes acute in the case of certain blood samples, can be solved by the addition of EDTA and butanol into the sample solution, and subsequent heating to about 70° at pH 1~2. The formation of Anhydroaureomycin due to heating at this pH was not observed.

3. The addition of sodium chloride for salting-out effect should be done after heating. If it is added during or before heating, the coagulation of protein will be irregular.

4. During the extraction procedure of Aureomycin from butanol into the thorium nitrate solution, it was found that the thorium extract was contaminated with a small amount of coloring substances, which showed absorption maximum at 400 mμ. Therefore, the method developed here for urine using the equation of McGillivary was not applied to blood samples. Subsequent studies on this procedure showed that the washing of thorium extract with butanol was effective. Table X shows that complete removal of interfering substances was reached with 5 cc. of butanol, whereas Aureomycin remained unchanged in thorium layer. The experimental data obtained are given in Table XI.

Table X.

Absorbance	Before washing		After washing	
	Blood	Serum	Blood	Serum
A 380 mμ	0.090	0.018	0.006	0.005
A 400 mμ	0.120	0.020	0.008	0.007
A 420 mμ	0.060	0.014	0.004	0.004

Table XI.

No. of washings	0	1	2
A at 380 mμ	0.184	0.163	0.170
A at 400 mμ	0.235	0.221	0.232

The authors wish to acknowledge the helpful advice and suggestions of Prof. M. Ishidate of the Pharmaceutical Institute, University of Tokyo.

Summary

An analytical method, which permits the application of thorium method reported previously by the authors, for determining Aureomycin in biological fluids was proposed. This method is based on the formation of Aureomycin-thorium complex which is soluble in water but insoluble in butanol. Aureomycin was effectively extracted from the sample with butanol when metals were masked with disodium ethylenediaminetetraacetate and salted out with sodium chloride at pH 1~2. The extracted Aureomycin in butanol was subsequently transferred to an aqueous medium in the form of Aureomycin-thorium complex by a thorium nitrate solution, and its absorbance was measured spectrophotometrically at 400 mμ, adjusted to pH 4.0~4.2 with sodium acetate-acetic acid buffer solution. By this method, as little as 2 γ/cc. of Aureomycin Hydrochloride can be successfully determined. The feasibility of employing this method for the determination of Aureomycin in urine and blood was demonstrated. The procedure seems to be adaptable to all forms of pharmaceutical preparations, and fermentation liquors, with minor modification.

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