Determination of Tetracycline Antibiotics by Alternating-Current Polarography

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A method for the analysis of tetracycline, chlortetracycline, and oxytetracycline by alternating-current polarography was developed. The method agrees favorably with microbiological assays and was shown to be applicable to medicinals containing these antibiotics. It was more sensitive and faster than direct-current polarography. Qualitative and quantitative determination of oxytetracycline in the presence of chlortetracycline appears to be possible. The effect of pH and degradation of these antibiotics on polarographic response in comparison to biological activity are discussed.

SEVERAL METHODS have been described for the determination of the tetracycline antibiotics. The microbiological methods are tedious and time consuming, and the chemical methods are, in general, not of adequate specificity. Chlortetracycline and oxytetracycline alone and in mixtures have been determined by direct-current (d.-c.) polarography (3–7, 14, 18).

Recently, Daftsios and Schall (2) applied alternating-current (a.-c.) polarography to the routine determination of 3,5-dinitrobenzamide and *N*-acetyl-*N*-(4-nitro-phenyl)-sulfanilamide. This suggested the possible use of a.-c. polarography for the routine determination of the tetracyclines.

A method was developed for the quantitative and qualitative determination of these antibiotics by a.-c. polarography. The determination of oxytetracycline in the presence of chlortetracycline also was demonstrated. The method was shown to be as accurate as conventional methods and can be applied easily to routine analyses of pharmaceuticals.

To the authors' knowledge, there have been no studies which utilize a.-c. polarography for the determination of the tetracyclines.

EXPERIMENTAL

Apparatus

A Sargent model XXI polarograph modified to record a.-c. polarograms, as described by Miller (12, 13), was employed. A Beckman pH meter, model 76 (expanded scale), and an electrolysis vessel of the mercury pool type were used. Size is not critical; however, a microtype vessel with a 3-ml. total volume was employed.

Reagents

Acid–Methanol Solution.—Two per cent H_3PO_4 (85%) in absolute methanol (v/v) (1:40).

Phosphate Buffer.—0.2 mole KH_2PO_4 per liter with pH adjusted to 4.1.

Chlortetracycline, oxytetracycline, and tetracycline antibiotic standards were the hydrochloride salts (99.5% pure samples).

Instrumentation

Calibration of the instrument is accomplished as described by Miller with some modifications (13). Obtain a pen deflection of 250 mm. using a 1000-ohm resistor with 10 mv. of a.c. and 0.0 mv. of d.c. applied with a sensitivity setting of 0.6 μamp./mm. After calibration, reset the sensitivity dial to an indicated 0.08 μamp./mm. (actual sensitivity = 0.0053 μamp./mm.) and span from −1.2 to −1.8 v.

Procedure

Antibiotic Standards.—Dry samples of antibiotic standards *in vacuo* at 60° for 3 hr.; then dissolve 100 mg. in 100 ml. of acid–methanol. Dilute aliquots (10 ml.) with phosphate buffer (50–75 ml.), adjust pH to 4.1, and bring to a final volume of 100 ml. with buffer. Make further dilutions with phosphate buffer to give concentrations of 20, 30, 50, and 60 mcg./ml. of the antibiotic.

Prepare a blank by mixing 10 ml. of acid–methanol with the appropriate amount of phosphate buffer and adjusting pH to 4.1.

Preparation of Sample.—Prepare commercial antibiotic preparations, such as tablets, capsules, and syrups and intravenous, intramuscular, and ear formulations, by dissolving the entire sample in acid–methanol with dilutions, if necessary, to yield a maximum solution concentration of approximately 1000 mcg./ml. Dilute aliquots (10 ml.) with phosphate buffer, adjust the pH to 4.1, and make to volume (100 ml.) with buffer. Make subsequent dilutions to bring the concentration within the 16–60 mcg./ml. range of the method.

For ointment preparations, dissolve an appropriate amount (1–5 Gm.) in petroleum ether. Shake the ether extract vigorously for at least 5 min. in a separator with each of four portions of phosphate buffer (50 ml.) and combine the washings. After pH adjustment, bring the solution to volume (250 ml.) with buffer. Make appropriate dilutions with buffer to bring the samples within the 16–60 mcg./ml. range.

Polarography.—Place 1 ml. of mercury in the electrolysis cell and add a sufficient quantity of antibiotic solution to cover the tip of the mercury capillary. Adjust the distance between the mercury

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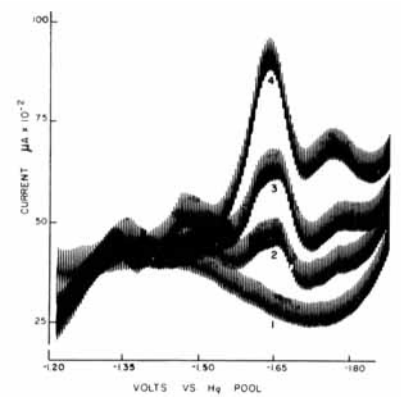


Fig. 1.—Alternating-current polarograms of chlortetracycline at pH 4.1. Key: 1, base; 2, 15 mcg./ml.; 3, 30 mcg./ml.; 4, 60 mcg./ml.

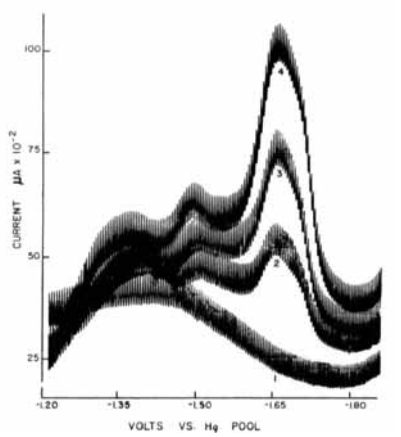


Fig. 2.—Alternating-current polarograms of oxytetracycline at pH 4.1. Key: 1, base; 2, 15 mcg./ml.; 3, 30 mcg./ml.; 4, 50 mcg./ml.

capillary and mercury pool to 7 mm. Using the above instrument calibration, record the polarogram from −1.2 to −1.8 v. and measure the total peak height at −1.65 v. at the midpoint of the pen oscillation. In each case, subtract the base current from the total height to yield the diffusion current, *i.e.*, peak height above the base line. Record the polarograms at room temperature (25°) without solution deaeration.

Microbiological Assays.—Assays were made using the technique described by Grove and Randall (6) with *Bacillus cereus* (ATCC 9634) as the test organism. The initial solution of the sample is the same as the polarographic preparation.

RESULTS

Examination of the polarograms obtained for each antibiotic (Figs. 1 and 2) shows that, although several reduction peaks are evident, only the one at −1.65 v. showed sufficient sensitivity in phosphate buffer at pH 4.1 for quantitative estimation. When antibiotic concentration was plotted *versus* diffusion current, a linear relationship was observed between 16 and 60 mcg./ml.

Qualitative identification of the antibiotic can be made easily at pH 4.1 due to the fact that chlortetracycline shows an additional response at −1.76 v. (Fig. 1) which is not exhibited by oxytetracycline (Fig. 2).

For the application of the a.-c. polarographic method of analysis to medicinals, simulated pharmaceutical preparations were prepared in the laboratory and analyzed. The objective was to investigate the recovery of the antibiotics from preparations containing known amounts of the antibiotic. In all preparations except syrups, chlortetracycline and oxytetracycline were added as the hydrochloride; in syrups, the dicalcium salt was used. The results are shown in Table I. The buffer salts and preservatives added to these preparations had no effect on the polarographic response.

Several commercial samples of the antibiotics were obtained to determine if commercial formulation had any significant effect on the analyses. Since the antibiotic content of these samples declared on the label may vary slightly from the actual amount of that incorporated into the sample, both polarographic and microbiological assays were carried out on each of the samples. The results (Table II) showed favorable agreement between the two methods. Therefore, no adverse effect upon the polarographic method is encountered due to commercial formulation.

TABLE I.—A.-C. POLAROGRAPHIC ANALYSES OF SIMULATED PHARMACEUTICAL PREPARATIONS

Prepn.	Antibiotic Added, mg.	Antibiotic Found, mg.	S.D.[a]
Chlortetracycline			
Ear soln.	50.0	50.7	0.98
i.v. soln.	500	509	6.52
Capsule	125	121	1.73
Syrup	500	508	4.62
Oxytetracycline			
Ophthalmic soln.	29.0	30.3	1.35
Capsule	135	132	3.71
Syrup	772	783	16.9

[a] Calculations based on six to ten separate samples.

TABLE II.—A.-C. POLAROGRAPHIC AND MICROBIOLOGICAL ASSAYS OF COMMERCIAL PHARMACEUTICALS

Prepn.	Antibiotic Declared, mg.	Antibiotic Found[a] a.-c. Polarog., mg.	Antibiotic Found[a] Microbiol., mg.
Chlortetracycline			
Ear soln.	50/vial	53.0	52.8
i.v. soln.	500/vial	504	490
Syrup	3000/bottle	3075	2672
Capsule	250/capsule	243	237
Ointment	30/Gm.	28.0	27.5
Oxytetracycline			
Ophthalmic soln.	25/vial	27.2	29.6
i.v. soln.	500/vial	525	561
Syrup	1500/bottle	1761	1665
Capsule	125/capsule	158	155
Ointment	30/Gm.	31.1	31.3

[a] Duplicate analyses of individual samples.

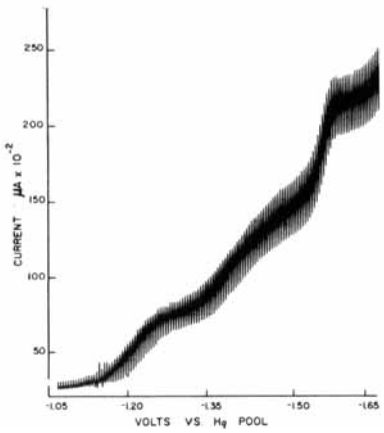


Fig. 3.—Direct-current polarogram of chlortetracycline or oxytetracycline at pH 4.1; concentration, 50 mcg./ml.

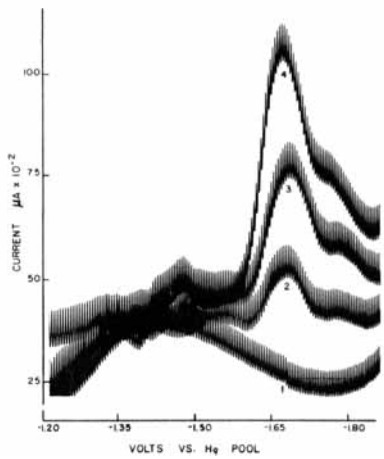


Fig. 4.—Alternating-current polarograms of tetracycline at pH 4.1. Key: 1, base; 2, 15 mcg./ml.; 3, 30 mcg./ml.; 4, 50 mcg./ml.

To compare the a.-c. polarographic method with the microbiological assay, two standard solutions each containing 500 mcg./ml. of the pure hydrochloride salt of the antibiotic were prepared in acid–methanol. Since degradation of the antibiotics is known to be slow when refrigerated (15, 16), the solutions were stored at 4°. Each solution was analyzed microbiologically and by a.-c. polarography at intervals for a period of 7 days. Since there was no apparent loss of potency, all results were averaged. As expected, both methods agreed favorably. Chlortetracycline assayed at 492 and 489 mcg./ml. and oxytetracycline at 498 and 493 mcg./ml. for polarographic and microbiological assays, respectively.

To show the advantage of a.-c. over d.-c. polarography, the d.-c. polarograms of chlortetracycline and oxytetracycline using the same buffer system were recorded. The d.-c. polarograms of the antibiotics under these conditions were identical (Fig. 3). Each solution contained 50 mcg./ml. of the antibiotic as the hydrochloride salt. Two clear reduction potentials were observed, and the reduction at a potential of approximately −1.56 v. could be utilized for quantitative estimation. The d.-c. polarographic results agree with those reported by other workers (3–5, 18).

The application of a.-c. polarography to the parent antibiotic, tetracycline, also was examined. The polarograms indicate that it was more responsive than either chlortetracycline or oxytetracycline (Fig. 4). A direct relationship between diffusion current and concentration was observed.

Operating Variables.—Higher or lower concentrations of the antibiotic solutions may be determined by varying the instrument calibration.

Higher concentrations (100–500 mcg./ml.) were handled by standardizing with the following procedure. A 250-mm. pen deflection was obtained, as mentioned previously, but with the sensitivity dial at 0.1 μamp./mm. The sensitivity dial then was reset after calibration to 0.06 μamp./mm.

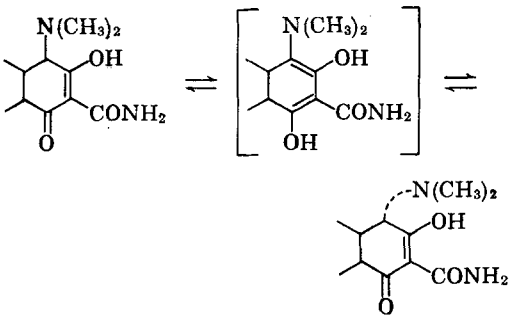
Lower concentrations (8–28 mcg./ml.) may be utilized using the following standardization accompanied by a recorder compensator adapted to a.c. according to Knevel (9). The calibration varies as follows. A 250-mm. pen deflection was obtained with the sensitivity dial at 0.8 μamp./mm. After calibration, the sensitivity dial is reset to 0.06 μamp./mm. with the down scale compensation adjusted to bring the baseline down scale in the spanned region of −1.20 to 1.80 v.

The distance between the dropping mercury electrode and the mercury pool should be kept constant for both standard and sample solutions. Varia-

Table III.—Biological *vs.* Polarographic Response During Storage of Antibiotic Solutions at 25°C.

Time, hr.	Phosphate Buffer, Antibiotic Found, mcg./ml. a.-c. Polarog.	Microbiol.	B.P.R.[a]	Acid–Methanol, Antibiotic Found, mcg./ml. a.-c. Polarog.	Microbiol.	B.P.R.
			Chlortetracycline HCl			
0	51.0	47.0	0.92	510	470	0.92
7	50.5	37.5	0.74	...	...	...
20	48.5	37.0	0.76	525	430	0.81
43	44.5	34.0	0.76	510	...	...
96	40.5	20.5	0.50	460	340	0.73
			Oxytetracycline HCl			
0	51.0	56.7	1.11	510	567	1.11
7	52.0	50.0	0.96	...	...	...
20	50.5	55.0	1.08	515	534	1.03
43	50.0	54.0	1.08	515	507	0.98
96	43.0	36.0	0.83	520	433	0.83

[a] B.P.R. = Biological activity/polarographic response.



Scheme I

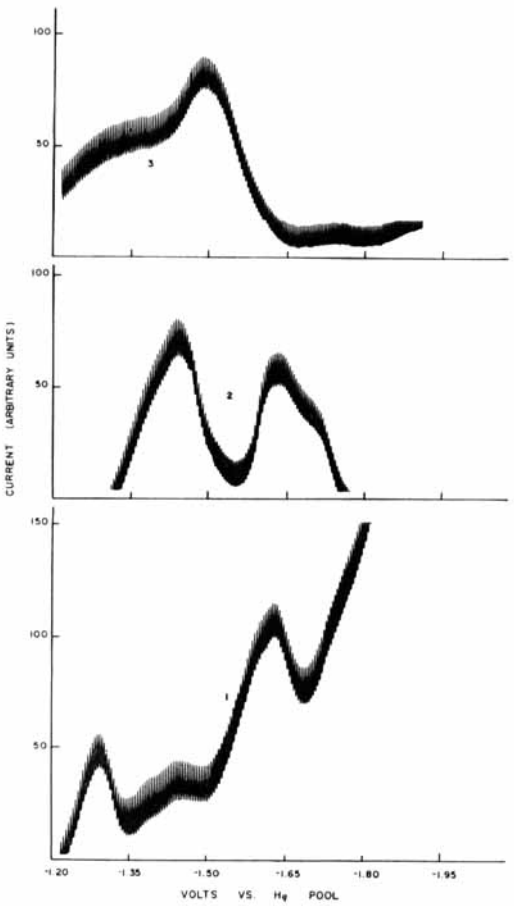


Fig. 5.—Alternating-current polarograms of chlortetracycline at various pH values. Key: 1, pH 3.30; 2, pH 7.44; 3, pH 9.2. Concentration, 50 mcg./ml.

tion in this distance relationship will cause a change in the height of the a.-c. polarographic reduction waves.

The stability of solutions stored at 4° has been shown already, and antibiotic solutions stored at this temperature are considered stable for use for approximately 1 week. It was of interest then to investigate the polarographic and biological activity of solutions stored at room temperature for various periods of time. Since degradation of the tetracyclines varies in aqueous and organic solvents (1, 8, 10, 11, 15), solutions of chlortetracycline and oxytetracycline were prepared in acid–methanol (500 mcg./ml.) and phosphate buffer (50 mcg./ml. and pH 4.15). These solutions were assayed periodically over a time interval of 96 hr. Results (Table III) showed that after 96 hr., biological activity decreased considerably, whereas polarographic response exhibited comparatively slight loss.

It is known that tetracyclines undergo epimerization in the pH range of approximately 2–6 (10). The equilibrium mixture is composed of approximately equal amounts of the respective tetracycline and its epimer. Since epimerization is known to be accompanied by loss of biological activity (8, 10, 11, 15, 17), this may explain the microbiological assay results. In regard to polarographic response, it is reasonable to assume that it is due to certain group(s) available for reduction (5). There is ample evidence in the literature (8, 10, 11) that epimerization at C-4 leads to the equilibrium depicted in the partial formulas shown in Scheme I.

It is possible that this epimerization was responsibe for the decrease of polarographic response. From the observed biological activity to polarographic

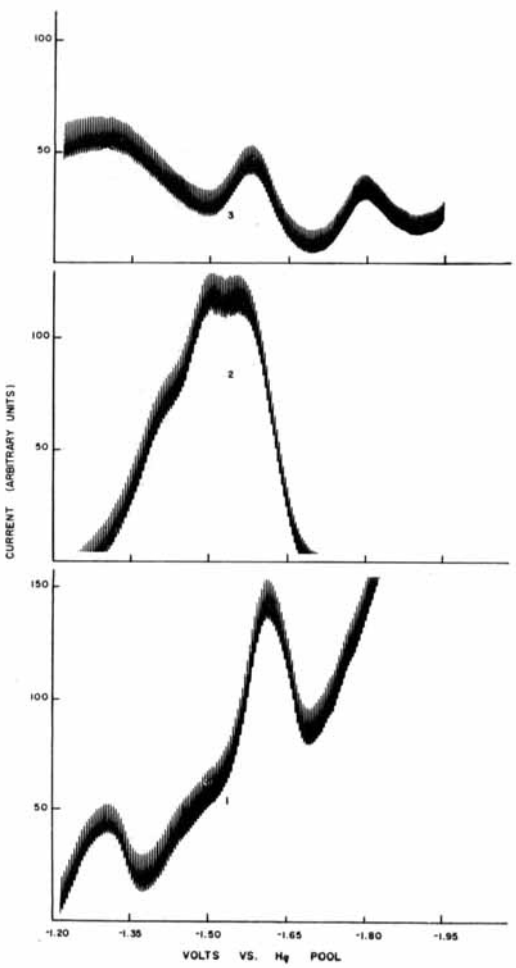


Fig. 6.—Alternating-current polarograms of oxytetracycline at various pH values. Key: 1, pH 3.27; 2, pH 7.32; 3, pH 9.11. Concentration, 50 mcg./ml.

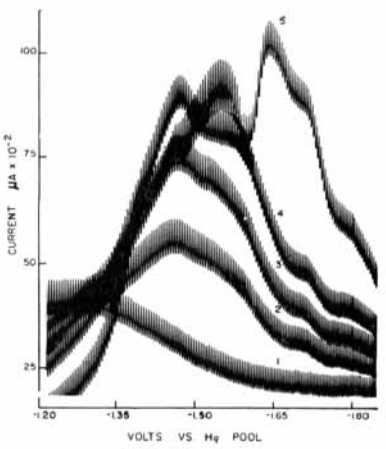


Fig. 7.—Alternating-current polarogram of a mixture containing equal amounts of oxytetracycline and chlortetracycline at pH 8.2. Key: 1, base; 2, 15 mcg./ml.; 3, 30 mcg./ml.; 4, 50 mcg./ml.; 5, 100 mcg./ml.

response ratios, one could assume that the same process which caused a decrease in antibiotic activity in the phosphate system—namely, epimerization—also was active in the methanol system but to a lesser degree. This agrees with other studies which reported tetracycline epimers form more readily in aqueous solutions, especially in the presence of buffers (10, 11). In view of these results, it is necessary to refrigerate the standard solutions (acid–methanol) when not in use and to assay the prepared sample solutions immediately following dilution to insure correlation with biological activity.

The pH had a profound effect on the characteristics of the a.-c. polarograms (Figs. 5 and 6). It was noted that at lower pH values (about 3.0) the recorded polarograms for both antibiotics are similar. This similarity begins to disappear as the pH is increased, and the difference is greatest at more basic pH values (about 9.0). The response for both antibiotics decreased as the pH was increased.

It should be mentioned that preparations of chlortetracycline at pH 6.7, when assayed immediately, gave 97% recovery. The stability of the antibiotic, however, is not optimum at pH values approaching neutrality (1), and they are not recommended for use.

It is interesting that between pH 8 and 9 the first peak (−1.40 v.) for oxytetracycline is absent, and a new reduction wave at about −1.80 v. is evident (Fig. 6). This reduction wave was not observed with chlortetracycline solutions (Fig. 5). Solutions containing mixtures of the antibiotics consistently showed a response at approximately −1.80 v. (variations in summit potential were observed with slight changes in pH), which was quantitative for oxytetracycline (Fig. 7). There are, however, limits to the analyses of these mixtures. The ideal mixture is one of equal amounts of the two antibiotics. As the concentration of chlortetracycline approached four times that of the oxytetracycline, the response due to oxytetracycline could not be resolved. However, within the limits described it is possible to determine oxytetracycline in the presence of chlortetracycline. Studies are now in progress to investigate the possibility of the quantitative determination of chlortetracycline in such mixtures.

The acid–methanol solution of syrups should be pipeted within 0.5 hr. after the solution is prepared. Longer periods of time allow the buffer salts in the preparation to crystallize, a process which removes a portion of the antibiotic from solution and gives lower recoveries.

CONCLUSION

Alternating-current polarography for analyses of the tetracyclines appears to offer a unique analytical tool. It is faster and more sensitive than d.-c. polarographic methods. It offers a possibility for further studies of metabolism of these antibiotics since metabolites, in all probability, would be inactive biologically or have different activities from the parent compound. Preliminary studies suggest that analyses of other antibiotics, such as bacitracin, penicillin, and streptomycin, are feasible.

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