In the mass spectrum, the highest mass ion was found at 615 and the base peak at 532 *m/e*. Although the material fragmented into a great many ions, the highest concentration of them clustered into two groups: one ranged from 530 to 536 and the other from 262 to 270 *m/e*. Since the starting material has a molecular weight of 302, the possibility that the ion at 615 *m/e* is the parent ion of a dimer of the starting material may be ruled out. The ion could possibly arise, however, from the fragmentation of a trimer formed from an intermediate generated during the reaction. The ions in the groups at *m/e* 530–536 and 262–270 could then be due to dimers and monomers, respectively, fragmenting from the trimers or present in the mixture obtained from the reaction.

Of the many structures possible, three monomeric ones that can be written for the ions and that appear to be consistent with the various spectral data and with the starting material are shown in IX, X, and XI.

Structures such as X and XI can arise from a rearrangement similar to that given for the dienol–benzene rearrangement (11).

It seems, therefore, that the initial reaction is a Friedel–Crafts reaction on the allylic alcohol group of I with the formation of an allyl carbonium ion. This carbonium ion can be stabilized not only by the excess aluminum chloride but also by the nitrobenzene (12, 13). The stabilized carbonium-ion complex, conjugated with one or more double bonds, results in the red color observed in the analytical reaction. Red oils were reported previously in reactions of aluminum chloride, or bromide, with aromatic hydrocarbons (14). When the complex is destroyed by the addition of water, polymeric material is formed by attack of the carbonium ions on the various double bonds initially present in the molecule or generated during the reaction.

REFERENCES

(1) K. Kato, *Chem. Pharm. Bull.*, **12,** 578(1964).

(2) *Ibid.*, **12,** 582(1964).

(3) H. Tauber, *Anal. Chem.*, **24,** 1494(1952).

(4) W. Lange, R. G. Folzenlongen, and D. G. Kolp, *J. Amer. Chem. Soc.*, **71,** 1733(1949).

(5) C. Liebermann, *Chem. Ber.*, **18,** 1803(1885).

(6) H. Burchard, *Chem. Zentral.*, **61,** 25(1900).

(7) L. Tschugaeff, *Chem. Ztg.*, **24,** 542(1900).

(8) A. Zlatkis, B. Zak, and A. Boyle, *J. Lab. Clin. Med.*, **41,** 486(1953).

(9) B. Zak, R. Dickenman, E. White, H. Burnett, and P. Cherney, *Amer. J. Clin. Pathol.*, **24,** 1307(1954).

(10) B. Zak, A. Boyle, and A. Slatkis, *Anal. Chem.*, **26,** 776(1954).

(11) E. B. Hershberg, *J. Amer. Chem. Soc.*, **80,** 3702(1958).

(12) G. B. Gill and G. H. Williams, *J. Chem. Soc., B*, **1966,** 880.

(13) F. R. Litterio, Ph.D. thesis, New York University, New York, N. Y., 1969, p. 62.

(14) G. A. Olah, "Friedel–Crafts and Related Reactions," vol. I, Interscience, New York, N. Y., 1963, p. 733.

ACKNOWLEDGMENTS AND ADDRESSES

Received November 30, 1970, from the *International Pharmaceutical Development Laboratory, Schering Corp., Bloomfield, NJ 07003*

Accepted for publication June 22, 1971.

Paper Chromatographic Determination of Oxytetracycline

A. SINA*, M. K. YOUSSEF†, A. A. KASSEM‡, and I. A. ATTIA

Abstract □ A paper chromatographic method for the quantitative separation and determination of oxytetracycline in the presence of its degradation products is presented. The separation of the antibiotic was carried out by making use of its complexation with urea on the chromatographic paper. Oxytetracycline and its degradation products gave characteristic fluorescence under UV light, and their respective R_f values were given. The recovery of this method was satisfactory (mean deviation = −3.0%) and was successfully applied to the assay of oxytetracycline in bulk and other pharmaceutical dosage forms. Hydrocortisone acetate and polymyxin B sulfate did not interfere with the chromatographic separation.

Keyphrases □ Oxytetracycline—separation, analysis from degradation products, urea-impregnated chromatographic paper □ Paper chromatography—separation, analysis, oxytetracycline □ Urea complexes—separation, analysis of oxytetracycline □ UV spectrophotometry—identification, oxytetracycline

The microbiological methods widely used for the determination of tetracycline antibiotics are not satisfactory, since some degradation products of these antibiotics proved to have antibacterial activity (1, 2). In view of the findings that relate a reversible fanconi-type syndrome to the ingestion of degraded tetracycline capsules (3–7), interest has developed in the analytical determination of tetracycline antibiotics in the presence of their degradation products.

Novelli *et al.* (8) pointed out the need of applying experimental correction factors, which are different for each component, in the spectrophotometric determination of tetracycline and its degradation products. This is necessary to make the results coincide with the theories and with the microbiological determinations.

A review of the literature dealing with the quantitative paper chromatographic determination of oxytetracycline revealed that there are only scanty publications concerning this aspect.

Selzer and Wright (9) separated different tetracyclines and their epimers on a paper chromatogram, previously impregnated with McIlvaine's buffer, pH 3.5. Sztaricskai (10) described a circular paper chromatographic method for determining oxytetracycline in the presence of its acidic decomposition products, using Whatman No. 1 chromatographic paper previously impregnated with phosphate buffer, pH 3.0, and a butanol–acetic acid–water (4:1:5) solvent system overnight. This method was claimed to be useful for estimating oxytetracycline in intermediary products and animal nutrients, but it was not reported to be applicable for the determination of oxytetracycline in pharmaceutical preparations. On applying this method to a sample of degraded oxytetracycline, we were unable to detect the fluorescent zones under UV lamp according to the description of the author; furthermore, no sharp separation of the degradation products was observed, and the experiment was time consuming (overnight running).

Higuchi and Bolton (11) reported that urea formed a complex with oxytetracycline and increased its solubility in buffer solution, pH 5.0. Based on this finding, it seemed valuable to use urea solution as an immobile phase for establishing a paper chromatographic method which allows separation and subsequent determination of oxytetracycline in the presence of its degradation products. The purpose of the present study is to establish an accurate procedure for the determination of oxytetracycline in different pharmaceutical preparations in the presence of its degradation products.

EXPERIMENTAL[1]

Separation of Oxytetracycline from Its Degradation Products—*Preparation of Chromatogram*—A series of chromatographic papers was impregnated with McIlvaine's buffer, pH 5.0. Other series of papers were impregnated with the same buffer containing 10% w/v of urea. The papers were then blotted between sheets of absorbent papers.

Solvents—A mixture of chloroform–ethyl acetate–pyridine (1:1:1), was used with papers impregnated with the buffer alone; the same solvent, previously saturated with urea, also was used with the papers impregnated with the buffer containing urea.

Method—Ten microliters from each freshly prepared methanolic solution, containing 1 mg./ml. of oxytetracycline base (international standard), and 10 μl. of the same solution after 1 month of storage at room temperature (34°) were separately spotted on each series of papers. The chromatograms were then partially dried in air; while still damp, they were subjected to ascending running by being hung in the chromatographic jars, each containing 100 ml. of the corresponding solvent. When the solvent front reached about 22 cm. above the starting line, the papers were removed and dried in air. They were then exposed to ammonia vapor for 5–15 sec., and the separated spots were identified under UV light. The oxytetracycline and its degradation products separated on the chromatogram were identified by analogy to the Chas. Pfizer test procedure (12). The mean R_f values of these components were calculated and are presented in Figs. 1 and 2.

Quantitative Analysis along a Horizontal Line—Two hundred microliters of a freshly prepared methanolic solution of oxytetracycline base was spotted along a horizontal line (10 cm.) on the chromatographic paper, previously impregnated with 10% w/v urea in McIlvaine's buffer, pH 5.0. Ten microliters of freshly prepared oxytetracycline (international standard) solution in methanol was spotted at the same level of the previously applied line at a distance 2.5 cm. away from the edge of paper. The chromatogram was then transferred to the chromatographic jar containing the corresponding, previously mentioned solvent. When the solvent front reached about 22 cm. above the starting line, the chromatogram was removed and dried in air. It was then exposed to ammonia vapor for the specified time and examined under UV light. The horizontal area at the level of the standard spot (yellow fluorescence) was cut off and then eluted with 20 ml. of dilute HCl (pH 1.8). The absorbance of oxytetracycline present in the eluate was determined spectrophotometrically at 352 nm. for 1 hr. against oxytetracycline base (international standard), which was chromatographed and eluted in the same manner. A blank for spectrophotometric determination was prepared by eluting an equal area from a chromatographic paper similarly treated without antibiotic. A series of chromatographic determinations was conducted on oxytetracycline (international standard) to calculate the percent recovery of the antibiotic (Table I).

Chromatographic Determination of Oxytetracycline in Different Pharmaceutical Preparations—The previously mentioned method was applied to determine the oxytetracycline content of capsules, tablets, injections, suspensions, pediatric drops, and ointments. The procedure adopted for preparing the sample before applying on the chromatographic paper was varied according to the nature of the dosage form used.

Capsules and Tablets—An exact weight, corresponding to 100 mg. oxytetracycline, from the pooled contents of five capsules or five powdered tablets was dissolved in a sufficient amount of methanol, and the volume was completed to 100 ml. The resulting turbid solution was centrifuged before being applied onto the chromatographic paper, and then 200 μl. of the methanolic clear solution was measured. The procedure was completed as previously described.

Suspensions and Pediatric Drops—An exact volume of the well-shaken sample, corresponding to 25 mg. oxytetracycline, was measured and diluted to 25 ml. with methanol in a volumetric flask. The resulting turbid solution was then centrifuged prior to applica-

Table I—Percent Recovery of Oxytetracycline (International Standard) after Chromatography

Number of Experiment	Oxytetracycline in 0.2 ml. Methanolic Solution, mcg.	Oxytetracycline Found after Chromatography, mcg.	Deviation	
			Micrograms	Percent w/v
1	200.0	197	−3	−1.5
2	200.0	192	−8	−4.0
3	200.0	193	−7	−3.5
4	200.0	195	−5	−2.5
5	200.0	194	−6	−3.0
6	200.0	195	−5	−2.5
7	200.0	192	−8	−4.0
		Mean =	−6	−3.0

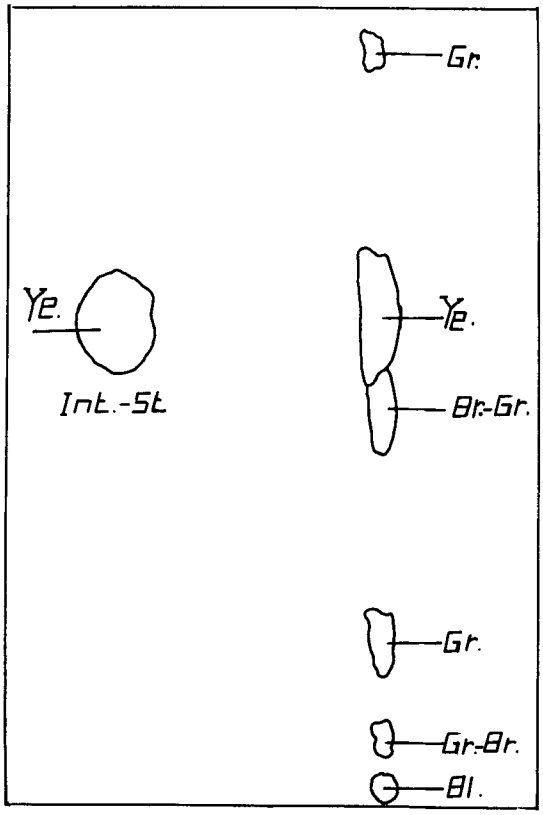

Figure 1—*Chromatogram of degraded oxytetracycline base on paper impregnated with buffer, pH 5.0. Key: Gr. (green) = α- or β-apoterramycin, R_f 0.95 or 0.2; Ye. (yellow) = oxytetracycline, R_f 0.61; Br.-Gr. (brownish-green) = terrinilide, R_f 0.49; Gr.-Br. (greenish-brown) = unknown, R_f 0.08; Bl. (blue) = 6β-deoxyoxytetracycline, R_f 0.02; and Int.-St. = international standard of oxytetracycline base dihydrate, R_f 0.61.*

[1] The following apparatus and reagents were used: (*a*) spectrophotometer, Carl Zeiss Jena; (*b*) UV lamp, HPW-125W, Typ. 57202 E/7, Philips; (*c*) chromatographic jars (20 × 25 × 35 cm.), with ground-glass lid; (*d*) Whatman No. 1 chromatographic papers (17.5 × 28 cm.); (*e*) methanol, chloroform, ethyl acetate, pyridine, urea, phosphoric acid, and hydrochloric acid, all analytical grade; (*f*) oxytetracycline base dihydrate USP XVII; and (*g*) international standard of oxytetracycline base dihydrate (900 I.U./mg.), provided by the World Health Organization.

Table II—Stated and Found Potency Values of Oxytetracycline in Different Pharmaceutical Preparations

Dosage Form	Stated Potency	Found Potency (Average of Four Determinations)	Standard Deviation
Capsules:			
No. 1	250 mg./capsule	250.22 mg./capsule	±3.44
No. 2	250 mg./capsule	248.28 mg./capsule	±3.15
Tablet	250 mg./tablet	219.45 mg./tablet	±2.38
Suspension	125 mg./5 ml.	115.10 mg./5 ml.	±0.55
Pediatric drops:			
No. 1	100 mg./ml.	97.04 mg./ml.	±0.87
No. 2	100 mg./ml.	100.70 mg./ml.	±0.46
Injections:			
No. 1	100 mg./2 ml.	100.25 mg./2 ml.	±1.08
No. 2	250 mg./2 ml.	260.77 mg./2 ml.	±1.46
Ointments:			
No. 1	30 mg./g.	30.23 mg./g.	±0.27
No. 2	5 mg./g.	4.02 mg./g.	±0.02
No. 3 (ointment base composed of 4% polysorbate in vaseline)	30 mg./g.	29.47 mg./g.	±0.01

tion. In the case of colored suspensions of calcium dioxytetracycline pediatric drops, the dye interfered with the chromatographic separation and determination. The dye was removed by centrifugation of an accurately measured volume of the supension, corresponding to 200 mg. oxytetracycline (as calcium salt), and subsequent washing of the precipitate with successive 5-ml. portions of distilled water. The precipitate, which was partially soluble in methanol, was made completely soluble by dissolving it in 10% w/v phosphoric acid (10 ml.) and completed up to 200 ml. with methanol. Then, 200 μl. of the solution was applied directly onto the chromatographic paper, and the assay was completed as described.

Injections—A measured volume (0.2 ml.), corresponding to 5 mg. oxytetracycline, was diluted to 5 ml. with methanol; 200 μl. was applied directly onto the chromatographic paper.

Ointments—An exact weight of the ointment, corresponding to 30 mg. oxytetracycline, was treated with the least amount (6 ml.) of petroleum ether (b.p. 60–80°). This solvent was chosen because the antibiotic is almost insoluble in it (13). The insoluble antibiotic was extracted by shaking in a separator with three, exactly measured, successive 5-ml. portions of 0.1 *N* HCl. The combined antibiotic–acidic solutions were then diluted with a further 15 ml. of methanol; 200 μl. was applied onto the chromatographic paper.

In the presence of a nonionic surface-active agent[2], the difficulty produced by emulsion formation, during extraction of the antibiotic, was overcome by centrifugation of the formed emulsion.

Hydrocortisone acetate and polymyxin B sulfate were found not to interfere with the chromatographic separation. The results obtained are presented in Table II. Each potency reported in the table represents the average of four determinations.

The oxytetracycline contents of oxytetracycline suspension and pediatric drops No. 1 were determined by the prescribed method after 14 days of storage at room temperature and then after a further 5 days subsequent to the latter period at 50°.

RESULTS AND DISCUSSION

Results obtained by impregnating the chromatographic paper with McIlvaine's buffer, having different pH values, revealed that pH 5.0 was the most suitable for the separation of oxytetracycline from its degradation products (Fig. 1).

Figure 2 shows the effect of urea on the R_f values of the antibiotic and its degradation products.

Figures 1 and 2 reveal that impregnation of the chromatographic paper with 10% w/v urea solution in McIlvaine's buffer, pH 5.0, gave a sharp and complete separation of oxytetracycline from its degradation products. On comparing the R_f value of each component in the presence and absence of urea (Figs. 1 and 2), it was found that the use of urea resulted in the lowering of the R_f values of the separated components. This lowering of the R_f values and the sharp separation were parallel with the results obtained by using

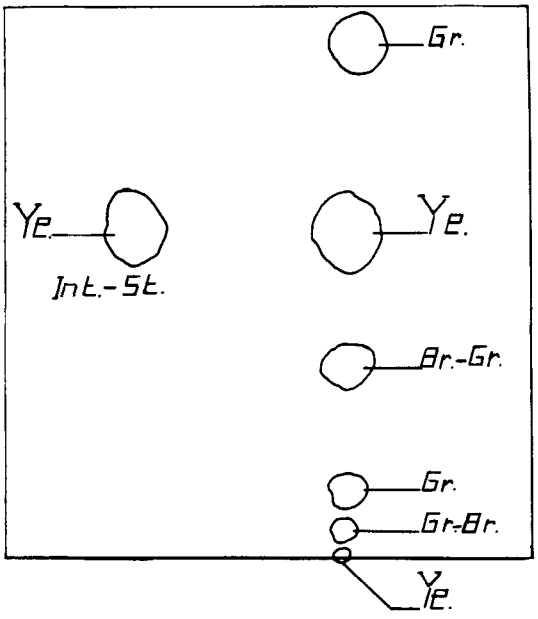

Figure 2—*Chromatogram of degraded oxytetracycline base on paper impregnated with buffer, pH 5.0, containing 10% w/v urea. Key: Gr. (green) = α- or β-apoterramycin, R_f 0.76 or 0.1; Ye. (yellow) = oxytetracycline or epiterramycin, R_f 0.48 or 0.01; Br.-Gr. (brownish-green) = terrinilide, R_f 0.28; Gr.-Br. (greenish-brown) = unknown, R_f 0.04; and Int.-St. = international standard of oxytetracycline base dihydrate, R_f 0.48.*

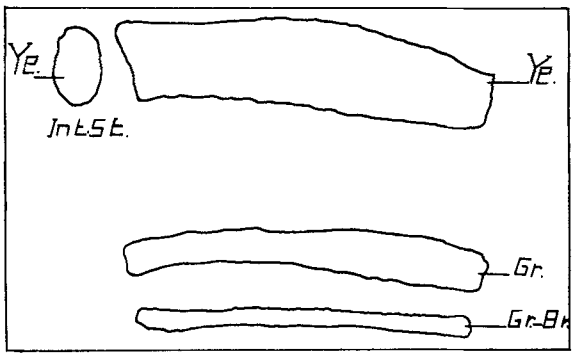

Figure 3—*Chromatogram of oxytetracycline base sample X_1 using horizontal line technique. Key: Ye. (yellow) = oxytetracycline; Gr. (green) = α- or β-apoterramycin; and Gr.-Br. (greenish-brown) = unknown.*

[2] Tween 20.

Table III—Microbiological and Paper Chromatographic Determinations of Oxytetracycline

Sample	Oxytetracycline Content, mcg./mg.		Standard Deviation (Chromatographic)
	Microbiological Method	Chromatographic Method	
X_1	820.0	790	±0.09
X_2	900.0	859	±2.88

edetic acid in the separation of tetracycline antibiotics (14). The influence of urea can be explained on the basis that urea forms a complex with the antibiotic on the chromatographic paper impregnated with buffer solution at pH 5.0 (11). The formation of oxytetracycline–urea complex on the paper chromatogram was confirmed by the authors by observing the shift toward the visible range of the spectrum in the maximum absorption of the antibiotic eluate, using McIlvaine's buffer, from the chromatogram previously impregnated with urea solution.

It was found that the application of more than 20 mcg. of the antibiotic per spot resulted in incomplete separation. To achieve a good quantitative separation, it would be necessary to apply at least 200 mcg. of the antibiotic on the chromatographic paper, using the horizontal line technique (Fig. 3), to provide a final concentration of about 10 mcg./ml. in the antibiotic eluate.

The percent recovery of oxytetracycline from the chromatogram (Table I) showed that the accuracy of the quantitative determination of this antibiotic by the prescribed method is adequate for its application in pharmaceutical preparations. The data indicated also that the antibiotic was not decomposed during the running time, as shown by the absence of degradation spots on the chromatogram of oxytetracycline (international standard). The stability of the antibiotic in the eluting solution (dilute HCl, pH 1.8) during the time needed for the spectrophotometric determination (1 hr.) was indicated by the absence of degradation spots on rechromatographing the acid eluate of the antibiotic. A further confirmation was carried out by observing the unchanged absorbances of the antibiotic acid eluate during 1 hr. The recovery losses presented in Table I were eliminated to some extent by using a reference standard similarly treated for each determination.

Table II clearly shows that the found potencies of the different dosage forms tested were nearly similar to the stated potencies, except with the oxytetracycline tablet and suspension. In the latter preparations, the number and the area of zones representing the degradation products of oxytetracycline were more than in the other dosage forms tested. Accordingly, the present method can be efficiently applied for determining oxytetracycline in pharmaceutical preparations.

The use of methanol with dilute hydrochloric acid in dissolving calcium dioxytetracycline, present in pediatric drops No. 2, prior to application on the chromatographic paper interfered with chromatographic separation. This unexpected observation may be attributed to the possible formation of a calcium chloride–oxytetracycline complex in the presence of hydrochloric acid (11), which in turn prevents the formation of oxytetracycline–urea complex on the chromatogram. This difficulty was overcome by using dilute orthophosphoric acid instead of hydrochloric acid.

Comparing the present method with the official microbiological technique, two different samples of oxytetracycline base dihydrate (X_1 and X_2), obtained from different manufacturers, were determined by both methods. The microbiological determination was carried out by using the cup plate method and *Bacillus* (var. mycoides, ATCC No. 9643) as a test organism (15). The results obtained are compiled in Table III. The values in the table are the averages of four determinations. From the table, it is obvious that there was an agreement between the prescribed method and the official microbiological procedure within 5% limits.

The present method proved to be useful in following the stability of oxytetracycline in different pharmaceutical dosage forms. It is obvious that oxytetracycline suspension is less stable than the pediatric drops (Table IV).

SUMMARY

1. The separation of oxytetracycline from its degradation products was quantitatively effected, using chromatographic paper previously impregnated by urea solution at pH 5.0.

2. The formation of an oxytetracycline–urea complex on the paper chromatogram was confirmed by observing the shift in the maximum absorption of oxytetracycline.

3. A quantitative paper chromatographic method is presented for the determination of oxytetracycline in bulk and in pharmaceutical dosage forms after separation from its degradation products.

4. The agreement of the presented method with the microbiological assay was discussed.

REFERENCES

(1) J. R. D. McCormick, S. M. Fox, L. L. Smith, B. A. Bitler, J. Reichenthal, V. E. Origoni, W. H. Muller, R. Winterbottom, and A. P. Doerschuk, *J. Amer. Chem. Soc.*, **79**, 2849(1957).

(2) J. L. Colaizzi, A. M. Knevel, and A. N. Martin, *J. Pharm. Sci.*, **54**, 1425(1965).

(3) J. M. Gross, *Ann. Intern. Med.*, **58**, 523(1963).

(4) G. W. Frimpter, *J. Amer. Med. Ass.*, **184**, 111(1963).

(5) L. I. Ehrlich and H. S. Stein, *Pediatrics*, **31**, 339(1963).

(6) *Ibid.*, **31**, 698(1963).

(7) S. M. Rosenthal, *Pediatrics*, **31**, 697(1963).

(8) G. Novelli, E. Superti, and C. Cattaneo, *Farmaco, Ed. Prat.*, **15**, 483(1960).

(9) G. B. Selzer and W. W. Wright, *J. Antibiot. Chemother.*, **7**, 292(1957).

(10) F. Sztaricskai, *Acta Pharm. Hung.*, **32**, 63(1962).

(11) T. Higuchi and S. Bolton, *J. Amer. Pharm. Ass., Sci. Ed.*, **48**, 557(1959).

(12) "Semi-Quantitative Determination of Anhydrotetracycline and Epi-Anhydrotetracycline in Tetracycline by Thin-Layer Chromatography," Test Procedure, Chas. Pfizer, Quality Control Department, Apr. 9, 1969, p. 4.

(13) P. J. Weiss, M. L. Andrew, and W. W. Wright, *J. Antibiot. Chemother.*, **7**, 374(1957).

(14) G. J. Kapadia and G. S. Rao, *J. Pharm. Sci.*, **53**, 223(1964).

(15) F. Kavanagh, "Analytical Microbiology," Academic, New York, N. Y., 1963.

ACKNOWLEDGMENTS AND ADDRESSES

Received September 9, 1970, from the *Faculty of Pharmacy, Pharmaceutics Department, Assiut University, Assiut, U.A.R.*

Accepted for publication June 22, 1971.

* El-NaSre Company for pharmaceutical chemicals and antibiotics, Cairo, U.A.R.

† To whom all correspondence should be addressed.

‡ Department of Pharmaceutics, Cairo University, Cairo, U.A.R.

Table IV—Stability of Oxytetracycline Suspension and Pediatric Drops

Dosage Form	Initial Concentration	Percent Remainder of Oxytetracycline[a]			
		After 14 Days at Room Temperature (34°)	Standard Deviation, %	After 14 Days at Room Temperature (34°) and 5 Days at 50°	Standard Deviation, %
Suspension	115.10 mg./5 ml.	82.97	±1.12	17.34	±0.32
Pediatric drops	97.04 mg./ml.	94.12	±0.64	69.29	±1.19

[a] Average of four determinations.