retical plate number. At room temperature, but not at 60°, a progressive decrease in flow rate is often experienced.

Plots of peak height *versus* amount of clindamycin 2-phosphate injected showed slight deviation from linearity at high sample charges (0.8–1.0 mg) (Fig. 5). Below 0.6 mg, excellent linearity is observed and peak heights can be used for quantitation.

DISCUSSION

The separation of clindamycin 2-phosphate from isomeric and related phosphate esters is sufficient to allow quantitation by the peak height approach.

Detection of clindamycin 2-phosphate by UV monitoring of background absorbance is remarkable and worthy of comment. The molar absorptivity of clindamycin 2-phosphate at 254 nm is only 16.8 in water at pH 8.90. Detection of clindamycin phosphate was made possible only by use of the high capacity support triethylaminoethyl cellulose. The exchange capacity of a 2.1-mm × 1-m column is 810–1160 μEq, as calculated from the column weight and the exchange capacity of the support. This value is approximately 20 times larger than the conventional liquid chromatographic ion-exchange supports which normally contain 48–60 μEq in the same size column (11). The use of high capacity supports should allow the detection of many compounds with low background absorbance at 254 nm.

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Simultaneous High-Speed Liquid Chromatographic Determination of Tetracycline and Rolitetracycline in Rolitetracycline Formulations

A. G. BUTTERFIELD [x], D. W. HUGHES, W. L. WILSON, and N. J. POUND

Abstract □ A rapid, precise high-speed liquid chromatographic procedure for the simultaneous determination of tetracycline and rolitetracycline in rolitetracycline formulations is described. Samples are dissolved in water, chilled to 0°, and chromatographed on a pellicular cation-exchange resin. The specificity of this method represents a significant improvement over present analytical procedures, which fail to differentiate between rolitetracycline and its hydrolysis product, tetracycline, in these formulations.

Keyphrases □ Rolitetracycline and rolitetracycline nitrate formulations—simultaneous high-speed liquid chromatographic analysis of tetracycline and rolitetracycline □ Tetracycline and rolitetracycline in rolitetracycline and rolitetracycline nitrate formulations—simultaneous high-speed liquid chromatographic analysis □ High-speed liquid chromatography—analysis, simultaneous, rolitetracycline and tetracycline in rolitetracycline and rolitetracycline nitrate formulations

Rolitetracycline (I) (2-*N*-pyrrolidinomethyltetracycline) is an antibiotic formed by *N*-aminomethylation of the carboxamide function of tetracycline (II). Compound I is formulated either as the base or nitrate salt for intravenous or intramuscular use. These formulations are intended to be made at the time of use with water suitable for injection.

The official method of assay of I in Canada (1) is microbiological. This method, however, gives no information on the extent of hydrolysis of I, since the test organism is also sensitive to II, nor any estimate of epimerization or degradation products expected to be formed under the assay conditions used. One would expect I, a Mannich base, to be susceptible to hydrolysis in aqueous solution, and Brunzell (2) demonstrated that aqueous solutions of I are indeed rapidly hydrolyzed with the formation of II and 4-epitetracycline (III).

By using high-speed liquid chromatography (HSLC), the stability of I was studied and it was confirmed that aqueous solutions are rapidly hydrolyzed to II (3). In addition, formulations of I were also found to contain II. Although the hydrolysis of I to II does not involve a loss of microbiological activity, the presence of II in formulations could be an indication of poor manufacturing practice.

This report describes a rapid HSLC procedure for the simultaneous assay of I and II in rolitetracycline formulations. Being precise and highly specific, the method represents a significant improvement over present analytical procedures for the quality control of rolitetracycline formulations.

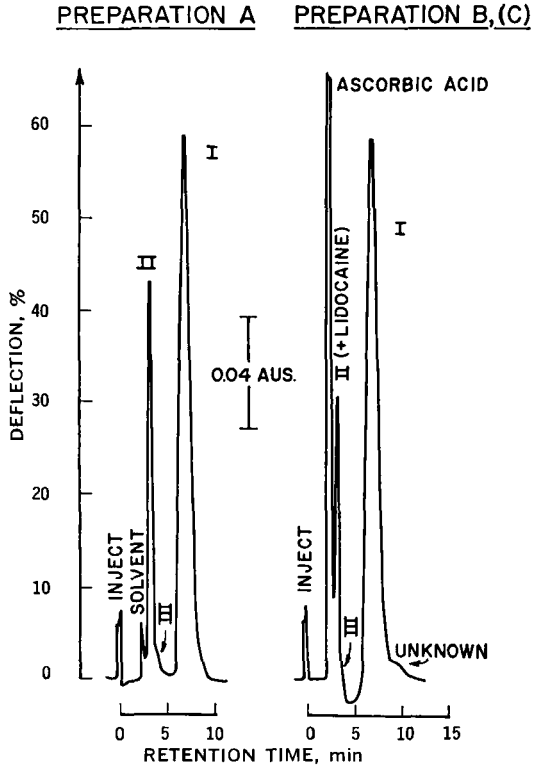

Figure 1—*High-speed liquid chromatograms of typical partially hydrolyzed rolitetracycline and rolitetracycline nitrate formulations. Key: A, rolitetracycline intravenous formulation; B, rolitetracycline nitrate intravenous formulation; and, C, rolitetracycline nitrate intramuscular formulation.*

EXPERIMENTAL

Materials—Rolitetracycline base (I)[1], rolitetracycline nitrate[2] (I-nitrate), tetracycline (II)[3], lidocaine hydrochloride[4], magnesium gluconate[5], and ascorbic acid[6] were used as obtained.

Apparatus—A liquid chromatograph[7] fitted with a constant-temperature water bath, a "septumless" injection port, and a fixed wavelength (254 nm) UV absorption detector (attenuated to 0.32 absorbance unit full scale) was used.

Chromatographic Parameters—A column (225 cm × 3.2 mm o.d. × 1.8 mm i.d., 304 stainless steel) was dry packed with a pellicular cation-exchange resin[8] by a procedure similar to that reported by Kirkland (4) and coiled (diameter, 25 cm) to fit horizontally into the water bath of the instrument. Each liter of mobile phase [0.10 *M* Na^+, 0.003 *M* ethylenediaminetetraacetate ($EDTA^{-2}$), and 40% ethanol; pH 4.35] was prepared by diluting a solution of ethanol (400 ml), 1.0 *M* sodium hydroxide solution (93.3 ml), and 0.05 *M* disodium ethylenediamine tetraacetate (66.7 ml) with water to a volume of 800 ml, adjusting to an apparent pH of 4.35 with acetic acid, and then bringing the solution to volume (1000 ml) with water.

Occasionally a small amount of flocculant precipitate formed during this procedure; however, it readily dissolved when the mobile phase was heated under reflux to remove dissolved gases and did not reprecipitate on cooling. The degassed solvent was stored in the solvent reservoirs of the instrument. A flow rate of 60 ml/hr (4100 psi) and an operating temperature of 25° were used throughout.

[1] Hoechst Pharmaceuticals, Montreal, Quebec, Canada.
[2] Bristol Laboratories of Canada, Candiac, Quebec, Canada.
[3] United States Pharmacopeia, Rockville, Md.
[4] Astra Pharmaceuticals (Canada) Ltd., Cooksville, Ontario, Canada.
[5] K&K Laboratories, Plainview, N.Y.
[6] British Drug Houses, Toronto, Ontario, Canada.
[7] Model 4100, Varian Aerograph, Walnut Creek, Calif.
[8] Pellionex CP-128, Northgate Laboratories, Hamden, Conn.

Table I—Labeled Composition of Rolitetracycline (I) and Rolitetracycline Nitrate Formulations

Formulation	I, mg/Vial	I-Nitrate, mg I/Vial	Magnesium Gluconate, mg/Vial	Ascorbic Acid, mg/Vial	Lidocaine Hydrochloride, mg/Vial
A_1[a]	275	—	434.5	—	—
A_2[a]	110	—	173.8	—	—
B[a]	—	350	273	575	—
C_1[b]	—	350	273	575	40
C_2[b]	—	150	109	246	40

[a] Intravenous formulations. [b] Intramuscular formulations.

Determination of Purity of Rolitetracycline Standard—Approximately 50 mg of the bulk drug standard of I was accurately weighed into a 2-ml volumetric flask. The sample was dissolved with shaking (10 sec), and the solution was immediately brought to volume with aqueous magnesium gluconate (43.4 mg/ml). The flask was placed in a water bath at 25°. Aliquots (0.25 μl) of the sample solution were withdrawn and injected, using a 1-μl syringe fitted with a reproducibility adaptor[9], at about 12-min intervals for a 3-hr period. The flask was shaken briefly prior to each sampling, and the time from solution makeup to elution of the peak due to I was recorded for each injection.

Determination of Synthetic Mixtures—*Rolitetracycline*—The desired amount of I was accurately weighed into a 2-ml volumetric flask. The sample was dissolved with shaking (about 10 sec), and the solution was brought to volume with aqueous magnesium gluconate (43.4 mg/ml). The flask was immediately placed in an ice bath (0°). The sample was shaken at 10-min intervals during the 30 min allowed for cooling. After 30 min, triplicate 0.25-μl aliquots were chromatographed.

Rolitetracycline Nitrate—The required amounts of I-nitrate and ascorbic acid (115 mg) were accurately weighed into a 2-ml volumetric flask. In the case of the synthetic intramuscular formulations, lidocaine hydrochloride (8.0 mg) was also added to the sample. The samples were then dissolved, brought to volume with magnesium gluconate solution, chilled, and chromatographed in the same manner as the synthetic mixture of I.

Determination of Commercial Products—*Drug Substances*—A sample of the drug substance (I or I-nitrate), equivalent to approximately 50 mg of I, was accurately weighed into a 2-ml volumetric flask, and the sample was treated by the procedure previously described for synthetic mixtures of I.

Formulations—The contents of a single vial (275 or 350 mg of I or I-nitrate) were dissolved in water and immediately transferred quantitatively to a 10-ml volumetric flask. In the case of 110- or 150-mg formulations, a 5-ml volumetric flask was used. The solution was then brought to volume with water, shaken, and placed in

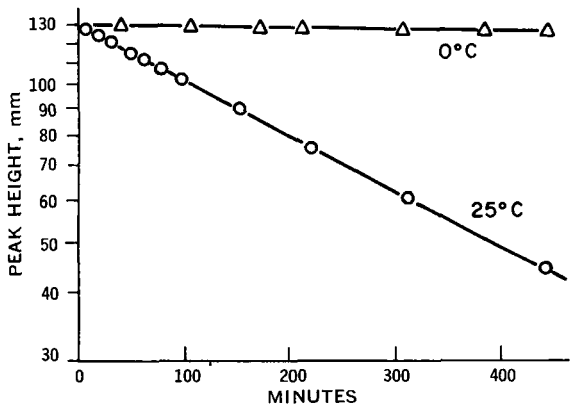

Figure 2—*Plot of log peak height* versus *time in solution from chromatograms of rolitetracycline solutions at two temperatures.*

[9] Model 7101N CH, Hamilton Co. Reno, Nev.

Table II—HSLC Analysis of Synthetic Rolitetracycline Formulations

Synthetic Formulations	Rolitetracycline (I)			Tetracycline (II)[a]			Total[a]		
	Calc., μg	Found, μg	Recovery, %	Calc., μg	Found, μg	Recovery, %	Calc., μg	Found, μg	Recovery, %
I	4.54	4.56	100.4	0.29	0.28	97	4.83	4.84	100.2
II	7.23	7.22	99.9	0.42	0.42	100	7.65	7.64	99.9
III	4.40	4.38	99.6	—	—	—	4.40	4.38	99.6
IV	5.43	5.42	99.7	—	—	—	5.43	5.42	99.7
Mean recovery, %			99.9			98			99.8
RSD, %			±0.4			±2			±0.3

[a] Expressed as equivalent amount of I.

Table III—HSLC Analysis of Synthetic Rolitetracycline Nitrate Formulations

Synthetic Formulations	Rolitetracycline (I)			Tetracycline[a] (II)			Total[a,d]		
	Calc., μg	Found, μg	Recovery, %	Calc., μg	Found, μg	Recovery, %	Calc., μg	Found, μg	Recovery, %
I[b]	3.43	3.41	98.6	0.17	0.17	100	3.60	3.58	99.5
II[b]	6.18	6.13	99.2	0.60	0.59	98	6.78	6.72	99.1
III[c]	3.68	3.66	99.5	0.93	0.95	102	4.61	4.61	100.0
IV[c]	4.23	4.15	98.1	0.80	0.81	101	5.03	4.96	98.6
Mean recovery, %			98.9			100			99.3
RSD, %			±0.6			±2			±0.6

[a] Expressed as equivalent amount of I. [b] Synthetic intramuscular formulation. [c] Synthetic intravenous formulation. [d] Total micrograms of I and II.

an ice bath. After cooling for 30 min, with shaking at 10-min intervals, triplicate 0.25-μl aliquots of the solutions were chromatographed.

Calculations—Concentrations of I and II were determined using the following expressions:

$$\text{I (micrograms per injection)} = (P_R + b)/m_R \quad \text{(Eq. 1)}$$

$$\text{II (micrograms per injection)} = [P_T - P_B - P_R(C)]/m_T \quad \text{(Eq. 2)}$$

$$\text{II (micrograms as I)} = \text{II (micrograms)} \times D \quad \text{(Eq. 3)}$$

where:

P_R = peak height for I (millimeters)
b = y intercept of calibration curve for I (− 5.7 mm)[10]
m_R = slope of calibration curve for I (24.1 mm/μg)[10]
P_T = peak height for II (millimeters)
P_B = peak height for sample blank (millimeters)
C = factor to correct for decomposition of I to II during sample preparation (0.061)[10]
m_T = slope of calibration curve for II (62.5 mm/μg)[10]
D = factor to express weight of II as an equivalent weight of I (1.187 = mol. wt. I/mol. wt. II)

RESULTS AND DISCUSSION

The high-speed cation-exchange chromatography system used in this analysis is a modification of one described in a previous study of the chromatographic behavior of a number of tetracycline derivatives (5). In the present investigation, the ethanol concentration of the mobile phase was increased to 40% and the operating temperature was reduced to 25° to minimize on-column hydrolysis of I.

Figure 1 illustrates the chromatograms obtained from the analysis of three, partially degraded, commercial formulations. Preparation A is made up of I and magnesium gluconate; while Preparation B contains I-nitrate, ascorbic acid, and magnesium gluconate. Preparation C is an intramuscular I-nitrate formulation containing lidocaine hydrochloride as well as ascorbic acid and magnesium gluconate. In addition to I and II, small amounts of III can also be detected in these degraded samples.

Under these conditions, retention times of 2.5, 3.1, 3.7, and 6.6 min were obtained for ascorbic acid, II, III, and I, respectively, while lidocaine hydrochloride and magnesium gluconate chromatographed as small peaks with retention times of 3.0 and 2.8 min, respectively. The latter two compounds are not resolved from the peak due to II; however, because of their low detector response, they are equivalent to less than 0.15 μg/injection of II in a typical formulation. A correction is made for the interference of these compounds by using a blank value (P_B) in Eq. 2.

Peaks due to two as yet unidentified substances, with retention times of 4.3 and 9.3 min, were also detected when I-nitrate preparations were chromatographed. One substance was present only in solutions containing ascorbic acid and produced a negative peak (4.3 min). A similar peak was also detected when aqueous solutions of ascorbic acid were chromatographed and is thought to be due to a displacement desorption of some other compound with a low UV extinction coefficient. The second substance (9.3 min) was present in all I-nitrate bulk drug samples and formulations examined. This peak may be due to 4-epirolitetracycline (IV). Neither of these peaks interferes with the determination of I or II in I-nitrate formulations.

Simultaneous quantitative analysis of both I and II is possible by relating peak height measurements to calibration curves for each of these two compounds. However, due to the intrinsic instability of solutions of I, it has been necessary to include correction factors in Eq. 2 to compensate for the formation of II from I during sample preparation.

The rate of degradation of I and I-nitrate is affected mainly by temperature (Fig. 2) and, to a lesser extent, by the concentrations of magnesium gluconate or of I in the sample solution[11]. Therefore, to facilitate accurate quantitative analysis and to permit the analysis of all formulations from a single calibration curve for I, samples containing a relatively high concentration of I (22–35 mg/ml) were prepared and immediately cooled to 0°. During the analysis, these samples were maintained at 0°. From Fig. 2 it can be seen that the rate of degradation is retarded to such an extent at 0° that any changes in the composition of the sample are negligible over the 1–2 hr required for an analysis. In fact, continued analysis of synthetic mixtures of I and I-nitrate over 7 hr at 0° showed less than 1.5% degradation.

The purity of the standard of I used throughout this study was determined relative to an FDA standard by UV (1) (99.4%), microbiological[12] (100%), and HSLC (100%) methods. HSLC was used to monitor the formation of II in three magnesium gluconate solutions of the standard of I at 25° over 3 hr. Extrapolation of the curve for II back to zero time indicated that no II was initially present in the sample; *i.e.*, it contained 100% I.

[10] Factors obtained under experimental conditions described here.

[11] Unpublished data.

[12] Reported by supplier.

Table IV—Analysis of Rolitetracycline (I) and Rolitetracycline Nitrate Formulations[a]

Sample	HSLC				Microbiological[e]	UV
	I	II[b]	IV[b,c]	Total[d]		
Rolitetracycline:						
110 mg, intravenous	105.6	1.3	—	106.9	111	106.3
275 mg, intravenous	110.9	0.7	—	111.6	114	110.3
Rolitetracycline nitrate:						
150 mg, intramuscular	104.0	4.2	14.0	108.2	112	122.2
350 mg, intramuscular	109.5	5.5	6.9	115.0	113	121.9
350 mg, intramuscular	113.3	6.9	13.7	120.2	118	133.9
350 mg, intravenous	111.9	7.1	7.4	119.0	115	126.4

[a] Results expressed as percent of labeled claim for I. [b] Expressed as equivalent amount of I. [c] By difference, UV minus Total. [d] Total I and II, *i.e.*, microbiologically active components. [e] Average of two determinations.

An absolute injection technique was used throughout this study. A relative standard deviation of 1.4% was obtained from the peak height measurements for the injection of five 0.25-μl (4.7-μg) aliquots of a sample of I maintained at 0°. Calibration curves were prepared for both I and II. A plot of peak height for I, against the amount of I injected for eight synthetic mixtures of I, was linear over a concentration range of 8–44 mg/ml (2–11 μg/injection) (Fig. 3). A least-squares analysis of the data showed that the linear portion of the curve could be expressed by an equation for a straight line, with a slope of 24.1 mm/μg and a y-intercept of −5.7 mm. Synthetic mixtures, prepared daily, were used throughout the study to check the calibration curve. The difference from the calculated values was never more than 1%.

A similar procedure was used for the determination of II in the formulations; however, the equation for the calibration curve for II was corrected for background interference caused by the presence of lidocaine hydrochloride or magnesium gluconate in the samples and for the amount of II formed from I during sample preparation. Analysis of six samples of pure I, cooled to 0° and left to equilibrate for 30 min, showed that a peak for II was obtained which was 6.1 ± 0.5% of the measured peak for I. Therefore, a factor of 0.061 (C, Eq. 2) multiplied by the measured I peak height (P_R, Eq. 2) was included to correct the measured II peak height for the formation of II during sample preparation and equilibration at 0°. Since measured I peak heights *versus* micrograms of I injected were plotted to obtain the calibration curve for I, no correction for decomposition during sample makeup was required in Eq. 1.

The calibration curve obtained from the analysis of solutions of II, in 0.01 M HCl, over a concentration range of 2–32 mg/ml (0.5–8 μg/injection) was a straight line through the origin with a mean slope of 62.5 mm/μg.

The value for the blank (P_B, Eq. 2) is readily obtained from the analysis of a blank solution containing the approximate concentration of ascorbic acid, lidocaine hydrochloride, and magnesium gluconate found in the formulation (Table I).

To facilitate correlation of the analytical data, it was convenient to express the weight of II as that weight of I from which it could have formed. Therefore, a third factor, 1.187 (D, Eq. 3), was included to convert the weight of II to the equivalent weight of I.

The results obtained from the analysis of synthetic formulations of I containing varying amounts of I and II are listed in Table II. Excellent correlation between the theoretical and observed values for the individual components and the total concentration of tetracyclines, expressed as I, was obtained. Similar correlation was observed between the HSLC and UV analyses of commercial formulations of I (Table IV).

The situation was somewhat more complex, however, in the case of I-nitrate and its formulations. The theoretical amount of I in pure I-nitrate bulk drug is 89.5%. Microbiological[12] and UV (1) analyses of I-nitrate bulk drug showed it to be pure (microbiological, 98%; UV, 102.5%). However, HSLC analysis of three replicates of the sample indicated that it contained 88.4 ± 0.3% of the expected amount of I along with an amount of II equivalent to 4.2 ± 0.2% of the expected amount of I. Thus, it can be concluded that the bulk drug contains at least one other UV-absorbing substance (about 7.4%) in addition to I and II. This observation is further substantiated by the fact that the HSLC chromatograms obtained for all I-nitrate bulk drugs and formulations contained an extraneous peak (9.3 min).

This extraneous peak was not due to the presence of 4-epianhydrotetracycline or anhydrotetracycline in the sample, since these compounds were found to have retention times of 8.6 and 4.9 min, respectively, under these conditions. It may, however, be due to IV-nitrate since it elutes after I. Previous work (5) showed that four 4-epitetracyclines were more retentive than the corresponding epimers. Furthermore, the microbiological assay results for the I-nitrate formulations (Table IV) are significantly lower than the UV results. This finding could be explained by the fact that during such an analysis IV would be hydrolyzed to III, which has been reported to possess approximately 5% of the microbiological activity of II (6). It has been shown[11] that I is more than 80% hydrolyzed in less than 30 min under the conditions specified for the microbiological assay. In addition, the formation of appreciable amounts of IV by the treatment of I with acetic acid was reported previously (7). When a sample of I was treated under these conditions and chromatographed, the size of the peak at retention time 9.3 min was increased to approximately two-thirds the size of the peak due to I. Finally, IV would be assayed by the UV method as II, since the tetracycline moiety is measured by this method. This would explain why the HSLC and UV methods agree for formulations of I, where none of this third compound is detected, and yet disagree where the compound is present, as in the I-nitrate formulations (Table IV).

The results from the analysis of a series of synthetic I-nitrate mixtures (Table III) showed good correlation between the calculated and found values for I and II in these mixtures. Mean recovery values of 98.9 ± 0.6 and 100.2 ± 2.8% were obtained for I and II, respectively. The UV analyses shown in Table IV indicate a higher concentration of total tetracyclines than the total amount of I and II determined by HSLC. This result is as expected, since the chro-

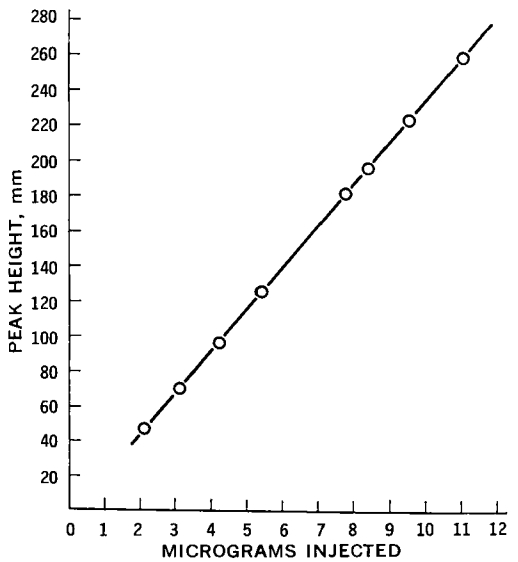

Figure 3—*Plot of peak height* versus *micrograms of rolitetracycline from chromatograms of rolitetracycline standard solutions.*

matogram of each of these mixtures possessed the small extraneous peak at 9.3 min.

The average amount of IV in the formulations (Table IV) was estimated, by difference, to be 10.5%. The UV analyses (mean = 126.1%) indicated an average "overfill" of 26.1%; therefore, the average amount of IV in the formulation is equivalent to 8.3% of the total tetracyclines, expressed as I. This value is comparable to the 7.4% found in the particular sample of I-nitrate bulk drug substance used in this study. Close correlation was also obtained between the results of the microbiological analyses and the total amount of I and II determined by the HSLC procedure. This finding is consistant with the fact that III, which would be formed by the hydrolysis of IV, is less microbiologically active than II (6).

In conclusion, the HSLC procedure provides a rapid, precise method for the simultaneous qualitative and quantitative analysis of rolitetracycline and its hydrolysis product, tetracycline, in both rolitetracycline and rolitetracycline nitrate formulations. A third component found in rolitetracycline nitrate formulations has not been identified conclusively, but UV and microbiological assays indicate that this compound is present at levels as high as 10% of total tetracyclines, contains a tetracycline moiety, and is comparatively inactive microbiologically. A tentative identification as 4-epirolitetracycline is suggested.

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PHARMACEUTICAL TECHNOLOGY

Versatile Unit for Filling Gelatin Capsules with Drugs or Chemicals

GEORGE R. THOMPSON [x] and ADAM CUNNINGHAM

Abstract □ A new, inexpensive device was developed for making individually prepared capsules that can be administered to experimental animals. The device was designed for accurate and rapid production of large numbers of various capsule sizes and drug dosages. During 12 months of usage, 21 capsules were prepared weekly for each of 44 dogs (924 total capsules) in approximately 2.5 workhours/week. Each capsule contained a precise amount of drug to administer a specific dosage to each individual dog. No difficulties were encountered in the manufacture or utilization of this device, and it can be operated by untrained personnel.

Keyphrases □ Capsules—device for making individually prepared capsules, design and application □ Gelatin capsules—rapid production of various capsule sizes and drug dosages, device described □ Equipment—rapid production of various capsules and drug dosages, design and application

Drugs and chemicals intended for human consumption or exposure must first be evaluated for toxicity in laboratory animals. The Food and Drug Administration and other governmental regulatory agencies recommend that the route of administration in animals corresponds to the route for proposed human usage (1). For subacute or chronic oral administration of food additives, pesticides, or other materials to rodents, the compounds can be incorporated into the diet. Dogs and monkeys can receive daily *per os* administration of drugs or other orally consumed chemicals by administration of the materials as tablets, in capsules, or by intubation of solutions or suspensions. Each of these three alternatives for drug administration involves significant shortcomings with large numbers of animals for long periods.

Manual production of tablets or capsules requires considerable effort and adds unduly to the cost of an experiment[1]. Preparation of tablets or capsules with commercial units decreases time and cost but sacrifices accuracy in dosage due to unit-to-unit variation (2, 3) and loss of the ability to adjust *precisely* for

[1] Manual preparation of 924 capsules/week for 44 dogs in a 1-year toxicity study could cost more than $15,800.