Tetracycline Degradation Products in Commercially Available Tetracycline-7-^{3}H

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Abstract □ The radiochemical purity of three commercially available tritium-labeled tetracycline products was investigated using TLC and spectrophotometry. All three products contained, in addition to tetracycline, substantial amounts of radioactively labeled 4-epitetracycline and anhydrotetracycline. Two products also contained an unidentified component which remained at the origin.

Keyphrases □ Tetracycline-7-^{3}H—radiochemical purity and identification of degradation products in three commercial samples □ Radiolabeled tetracycline—radiochemical purity and identification of degradation products in three commercial samples □ 4-Epitetracycline, radiolabeled—identified as degradation product in commercial tetracycline-7-^{3}H □ Anhydrotetracycline, radiolabeled—identified as degradation product in commercial tetracycline-7-^{3}H □ TLC—analysis, radiochemical purity of tetracycline-7-^{3}H

The chemical purity of commercially available tetracycline products was the subject of several recent investigations (1–5). In these studies, various analytical techniques revealed that virtually all products investigated contained, in addition to tetracycline (I), varying small amounts of tetracycline degradation products such as 4-epitetracycline (II), anhydrotetracycline (III), and 4-epianhydrotetracycline (IV). In view of these findings, the question arose whether commercially available, radioactively labeled I might also contain tetracycline-like impurities. The presence of such radiolabeled contaminants could result in serious errors in biological studies in which the antibiotic is assayed by nonspecific radioisotopic methods.

The purity of tetracycline-7-^{3}H obtained from two commercial suppliers was investigated using TLC and radiochromatographic scanning of TLC plates. These methods allowed estimation of the relative amounts of I, II, and III.

EXPERIMENTAL

Reagents—Analytical grade reagents used were disodium ethylenediaminetetraacetate, methanol, glycerin, polyethylene glycol 400, succinic acid, citric acid, *n*-butanol, and methyl ethyl ketone.

Solvent Systems—Two solvent systems were used: A, methyl ethyl ketone saturated with McIlvaine's buffer (pH 4.7)(6); and B, *n*-butanol–methanol–10% citric acid (4:1:2). It was previously reported (6) that I does not undergo degradation during TLC with Solvent System A.

Thin-Layer Plates—Kieselguhr G[1] and kieselguhr N[2] TLC plates were prepared as described by Gyanchandani *et al.* (6). Acid-washed silica gel[3] and silica gel[4] plates were pretreated by soaking for 1 hr. in a 0.1 *M* solution of disodium ethylenediaminetetraacetate and then were air dried for at least 16 hr. before use.

[1] Merck.
[2] Macherey, Nagel and Co.
[3] Acid-washed silica gel (ITLC-SA) instant thin-layer plates, Gelman Instrument Co.
[4] Silica gel (ITLC-SG) instant thin-layer plates, Gelman Instrument Co.

Table I—TLC Analysis of Nonlabeled Reference Compounds[a]

Compounds	R_f Values—Acid-Washed Silica Gel—Solvent System A[b]	R_f Values—Acid-Washed Silica Gel—Solvent System B[c]	R_f Values—Silica Gel—Solvent System B
I	0.51	0.52	0.87
II	0.30	—	—
III	0.95	0.85	0.99

[a] Compounds dissolved in Gomori's succinate buffer, pH 4.65. [b] Methyl ethyl ketone saturated with McIlvaine's buffer, pH 4.7. [c] *n*-Butanol–methanol–10% citric acid (4:1:2).

Nonlabeled Reference Compounds—I-Hydrochloride (I-HCl)[5] was used as the tetracycline reference standard. Compound II, the carbon-4 isomer of I, was prepared according to McCormick *et al.* (7). The III-base was prepared by the method of Simmons *et al.* (8).

Radioactive Materials—Two samples of I-HCl, labeled with tritium at C_7 (V and VI)[6], and a single lot of I-base, similarly labeled (VII)[7], were studied.

Detection—Nonlabeled reference compounds (I, II, and III) were visualized on TLC plates by their fluorescence in longwave UV light[8]. Tritium-labeled V, VI, and VII, along with radiolabeled degradation products, were detected using a radiochromatogram scanner[9]. All radiochromatogram scans were quantitated by tracing onto graph paper, cutting out the tracing, and expressing the weight of individual peaks as a percentage of the weight of the entire tracing.

RESULTS

Nonlabeled Reference Compounds—Twenty-five-microliter aliquots of 0.06% solutions of I, II, and III, dissolved in pH 4.65 Gomori's succinate buffer, were manually streaked in a band 2 cm. from the bottom and 1 cm. from each edge of 5 × 20-cm. acid-washed silica gel[3] or silica gel[4] plates, using one compound per plate. Immediately after drying, the plates were allowed to develop 14–15 cm. (45–50 min.) in Solvent Systems A and B at room temperature. The R_f values obtained are given in Table I. It can be seen that I, II, and III applied to the acid-washed silica gel plates[3] were well separated by Solvent System A. Solvent System B provided good separation of I and III but failed to distinguish these compounds from the C_4-epimer, II. Good separation of I and III was also obtained using the silica gel plates[4] and Solvent System B, but again II was not resolved.

Product V—One millicurie (1.2 mg.) of this product was received in the dry state. The material was dissolved in 1 ml. of methanol, appropriate volumes (50–100 μl.) of solution were transferred to siliconized[10] 1-dram vials, and the vial contents were taken to dryness *in vacuo* at room temperature. Vials were then sealed, protected from light, and stored at room temperature.

For TLC, a vial containing 50–100 μc. of Product V was dissolved in Gomori's succinate buffer (pH 4.65), distilled water, or methanol containing sufficient I-HCl to give a final concentration of 48 mcg./ml. Twenty-five-microliter amounts of these solutions were ap-

[5] Lot No. 174-009, 96.9% pure, Lederle Laboratories.
[6] Lot No. 268-238, specific activity 400 mc./mmole (V), and Lot No. 566-135, specific activity 500 mc./mmole (VI), New England Nuclear Corp.
[7] Lot No. WR-1159, specific activity 9 c./mmole, Schwarz/Mann.
[8] Chromato-vue, Ultra-violet Products, Inc.
[9] Model 7201, Packard Instrument Co.
[10] Siliclad, Clay Adams.

Table II—R_f Values and Percent Composition[a] of Product V as Determined by TLC[b]

Support Medium	Solvent	Unknown R_f	Unknown %	II R_f	II %	I R_f	I %	III R_f	III %	Total[c], %
Acid-washed silica gel	Buffer[d]	0	6.6	0.31	18.8	0.53	59.6	0.94	10.1	95.1
Acid-washed silica gel	Water	0	4.8	0.28	16.2	0.53	60.7	1.00	11.1	92.8
Acid-washed silica gel	Methanol	0	4.4	0.28	20.1	0.53	57.2	0.96	18.1	99.8
Kieselguhr G	Buffer	0	13.7	0.26	11.9	0.62	57.6	1.00	11.9	95.1
Kieselguhr N	Buffer	0	7.0	0.41	19.7	0.75	53.8	1.00	10.4	90.9

[a] Components identified by reference to nonlabeled reference compounds (Table I) and quantitated as described under *Experimental*. [b] Solvent System A (see Table I). [c] Total area of peaks on basis of weight and exclusive of background. [d] Gomori's succinate buffer, pH 4.65.

plied to 5 × 20-cm. acid-washed silica gel[3], kieselguhr G, and kieselguhr N plates by streaking, and the plates were developed to 14–15 cm. in Solvent System A. As shown in Table II, Product V was resolved by Solvent System A and acid-washed silica gel[3] plates into four areas of radioactivity. Whether Gomori's succinate buffer, water, or methanol was used as the initial solvent, the resulting chromatograms were qualitatively similar. Three radioactive peaks were identified as I, II, and III by reference to chromatograms of nonlabeled reference compounds. The results suggest that Product V is composed of 57–61% I, 16–20% II, 10–18% III, and 4–7% unknown material with zero R_f.

Results obtained with kieselguhr plates and Solvent System A compared favorably with those given above, except that there was a higher percentage of unknown material at the origin with kieselguhr G.

Since the tetracyclines have the property of forming chelates with certain metal ions, the material at the origin might be a complex of this nature. However, despite the presence of disodium ethylenediaminetetraacetate, a peak at the origin was consistently observed, even with kieselguhr N, which lacks a binding agent. If the radioactivity localized at the origin were a bound form of Product V, under- or overstreaking with nonlabeled I should reduce or eliminate this peak. However, spotting 0.3 mcg. of V in methanol on acid-washed silica gel, alone and before or after applying 100 mcg. of nonlabeled I, resulted in virtually identical chromatograms when developed in Solvent System A. The resulting chromatograms all contained four areas of radioactivity representative of 51–54% I (R_f 0.49–0.52), 17–21% II (R_f 0.25–0.28), 14–18% III (R_f 0.93–0.95), and 3–5% unknown material which failed to migrate.

The nature of this material at the origin has not been determined, but it is either firmly bound or very polar since it could not be eluted with methanol. Although elution was accomplished using Solvent System B, rechromatography of the eluted material on acid-washed silica gel with Solvent System A produced a single radioactive peak which remained at the origin.

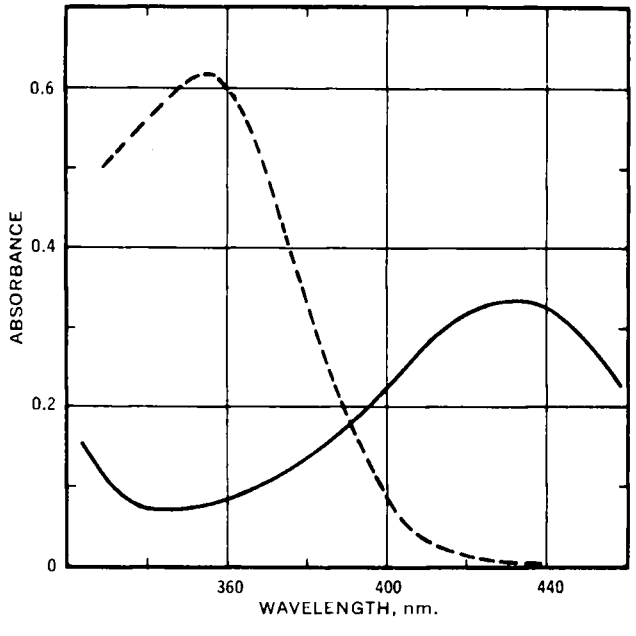

Figure 1—*Spectrophotometric curves for I-HCl (- - -) and Product VI (——). Each solution contained 20 mg. of compound/l. of 0.1 N hydrochloric acid.*

Product VI—One millicurie (1 mg.) of VI, as received in the dry state, was dissolved in 1 ml. of methanol, and 50 μl. of a 1:200 dilution in methanol was streaked on acid-washed silica gel and chromatographed in Solvent System A. One-tenth-milliliter aliquots of the remaining initial methanol solution were pipeted into 1-dram vials and treated as described previously for Product V. The vial contents were reconstituted by adding 1 ml. of methanol, and chromatography was accomplished by spotting 5 μl. (0.5 μc.) of this solution on acid-washed silica gel and silica gel and developing in Solvent Systems A and B, respectively. Methanol solutions of I-HCl and III (1 mg./ml.) were spotted in 5-μl. aliquots on the same plates.

Chromatograms of the initial solution of Product VI (before it was divided and taken to dryness) produced three areas of radioactivity (Table III). Two radioactive peaks were identified as I (R_f 0.50) and III (R_f 0.90) by reference to results obtained simultaneously with nonlabeled reference compounds. The third peak of radioactivity remained at the origin. The major radioactive component had the most rapid migration rate and was localized at an R_f value corresponding to that obtained with III.

Chromatograms of Product VI taken to dryness and reconstituted with methanol also produced three areas of radioactivity (Table III). As with the initial solution, a minor peak was present at the origin; the remaining peaks were identified as I (R_f 0.51) and III (R_f 0.94). Once again, the major radioactive component was localized at an R_f value corresponding to that obtained with III.

When reconstituted Product VI was chromatographed on silica gel with Solvent System B (Table III), a single radioactive peak was obtained with an R_f value equal to 0.98, the value expected with III in this system. Thus, TLC of both initial and reconstituted methanol solutions of Product VI on acid-washed silica gel and silica gel with Solvent Systems A and B suggested that this product is mainly III rather than I.

To confirm the chromatographic results, Product VI was assayed spectrophotometrically[11] using the absorbance ratio method described by Pernarowski *et al.* (9). Seven and one-half milliliters of 0.1 *N* hydrochloric acid was added to a vial of Product VI containing 150 mcg. of material. The spectral characteristics of this solution were determined by scanning from 320 to 460 nm., using 0.1 *N* hydrochloric acid as a blank. The spectral characteristics of I-HCl (20 mg./l. of 0.1 *N* hydrochloric acid) were determined in the same manner (Fig. 1). All scans were completed within 3 hr. after solution preparation.

The spectrophotometric curve obtained with I-HCl indicated an absorption maximum at 357 nm., in agreement with results of Pernarowski *et al.* (9). In contrast, the absorption maximum obtained with Product VI was at 434 nm., the wavelength of maximal absorption for III and IV (9). Furthermore, the absorbance of solutions of I-HCl and Product VI were equal (A_s = 0.178) at 391 nm., the wavelength at which I, III, and IV have identical absorptivity values (a_s) (9).

The absorbance of the solution of Product VI at 357 and 391 nm. was used with the following equation (9) to calculate the percentage of I present in the solution:

$$\%\ \mathrm{I} = \frac{Q{:}357{:}391 - 0.23}{0.0367} \qquad \text{(Eq. 1)}$$

in which Q:357:391 represents the absorbance ratio value, while

[11] Beckman DU-2.

Table III—TLC Analysis of Product VI

	R_f Values					
	Acid-Washed Silica Gel—Solvent System A[a]				Silica Gel Solvent System B[a]	
Product VI (initial solution)[b]	0.01	—	0.50	0.90[c]	—	—
Product VI (reconstituted)[d]	0.02	—	0.51	0.94[c]	—	0.98[c]
Nonlabeled reference compounds[e]						
I		—	0.51	—	0.87	—
II	—	0.29	—	—	—	—
III	—	—	—	0.91	—	0.99

[a] As described in Table I. [b] Product VI, as received in the dry state, dissolved in methanol. [c] Major radioactive component. [d] 1:10 methanol dilution of initial solution of Product VI. [e] Methanol solutions, 1 mg./ml.

0.0367 and 0.23 are the slope and y-intercept, respectively, of the equation for the linear relationship between Q values and percent I in various synthetic mixtures. By using Eq. 1, the relative concentration of I in the solution of Product VI was calculated to be only 6.1%, while application of Eq. 1 to the absorbance values obtained with a solution of nonlabeled I-HCl indicated the presence of 88% I.

Product VII—This product was received as a solution containing 1 mc. (53 mcg.) in 2 ml. of ethanol. The solution was stored in a sealed vial at 0° and samples were taken as needed.

For chromatography, 50 μl. of Product VII was diluted to 1 ml. with methanol or Gomori's succinate buffer containing nonlabeled I. Twenty-five-microliter quantities of these solutions were applied to acid-washed silica gel plates and developed in Solvent System A (Table IV). Product VII was resolved into three areas of radioactivity. The R_f of the major peak was the same as that of I, and the R_f values of the two minor peaks were the same as those of II and III (Table I). Unlike Products V and VI, Product VII did not show a radioactive peak at the origin when chromatographed in an identical manner.

DISCUSSION

All three samples of commercially available tritiated tetracycline were found to contain substantial amounts of radioactive degradation products. In fact, chromatographic and spectrophotometric studies suggested that Product VI is almost entirely a mixture of III and IV. Products V and VII contained 50–60% I together with smaller amounts of II and III. In addition, Products V and VI contained labeled material that remained at the origin after TLC. The amount of this material was not significantly altered by understreaking or overstreaking the site of application with a solution of nonlabeled I, as might be expected if the material were a bound form of I. It is possible that this material is chemically different from bound I or is a decomposition product of I other than those resulting from epimerization.

Gyanchandani *et al.* (6) reported that when I and its degradation products were chromatographed on kieselguhr G using Solvent System A, I and IV migrated at nearly the same rates with R_f values of 0.53 and 0.47, respectively. In the present study with acid-washed silica gel plates and Solvent System A, I and IV were not resolved into separate radioactive peaks. Therefore, the radioactive peaks identified in this study as I probably represent a summation of the percentages of I and IV, in which case the percentages of I listed in the tables would be maximal values. Since epimerization results in an equilibrium mixture (7), the presence of relatively small proportions of III means that a correspondingly small fraction of IV will be present also. Conversely, when larger proportions of III are present, the proportion of IV should be correspondingly greater. The latter situation appears to exist in the case of Product VI.

Table IV—R_f Values and Percent Composition[a] of Product VII as Determined by TLC[b]

Diluent	II		I		III		Total[c], %
	R_f	%	R_f	%	R_f	%	
Methanol	0.27	17.5	0.51	51.4	0.94	23.7	92.6
Buffer	0.31	15.9	0.53	52.5	0.95	24.9	93.3

[a] Components identified by reference to TLC of nonlabeled reference compounds and quantitated as described under *Experimental.* [b] Original ethanol solution diluted with methanol or Gomori's succinate buffer, pH 4.65, and chromatographed on acid-washed silica gel with Solvent System A (see Table I). [c] Total area of peaks on basis of weight and exclusive of background.

Whereas chromatographic analysis of reconstituted Product VI indicated the presence of about 25% I, the calculated amount of I present as determined spectrophotometrically was 6.1%. Apparently about 19% of the material in Product VI that has an R_f value corresponding to I is actually IV. With only 6.1% of I present, it is unlikely that detectable amounts of II would be formed by epimerization; this could account for the apparent absence of II from Product VI.

Biological tracer studies with labeled compounds that contain radioactive degradation products may be misinterpreted if the investigator is unaware of the presence of such contaminants. For example, the labeled isomeric forms of I present in Products V, VI, and VII cannot be assumed to be handled by the body in a manner identical with I. Therefore, the use of such a product in a pharmacodynamic study would necessitate using a method of assay that could quantitatively distinguish among the various components of the radioactive product. Such a requirement greatly reduces the advantages of using labeled I over nonlabeled I.

Results of this study underline the importance of verifying the radiochemical purity of commercially available labeled compounds using analytical systems that have been demonstrated to resolve isomers and degradation products adequately.

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