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ˣ To whom inquiries should be directed.

Influence of pH and Route of Injection on Acute Toxicity of Tetracycline in Mice

ROBERT T. WIVAGG, JAMES M. JAFFE, and JOHN L. COLAIZZI ˣ

Abstract □ Some LD_{50} determinations for tetracycline hydrochloride in mice were carried out over a range of pH values, using both the intraperitoneal and the subcutaneous routes of injection. Depending on the pH of the formulation, either water or a solvent system of water and 60% (v/v) propylene glycol was employed thus ensuring complete solution of the drug at all pH values tested. Higher LD_{50} values were obtained with the subcutaneous route than with the intraperitoneal route. With either route of administration, there was a trend toward lowest LD_{50} value occurrence at the isoelectric pH of tetracycline. Actual statistical significance was achieved for the intraperitoneal route only when LD_{50} values obtained at the isoelectric pH were compared with either of the more acidic pH values.

Keyphrases □ Tetracycline—effect of pH and route of injection on acute toxicity, mice □ pH—effect on acute toxicity of tetracycline, mice □ Injection route—subcutaneous *versus* intraperitoneal, effect on acute toxicity of tetracycline, mice □ Toxicity—tetracycline, effect of pH and route of injection, mice □ Antibiotics—tetracycline, effect of pH and route of injection on acute toxicity, mice

The acute toxicity of a drug is usually defined in terms of an LD_{50} value, which is the milligrams per kilogram dose of a drug that, on the average, will kill one-half of the group of test animals of a certain species under specified and controlled conditions following administration of a single dose (1). In the required acute toxicity testing performed on new drug entities, two factors that can influence the experimental results with a parenterally administered drug are its formulation and route of injection (2). For example, it has been demonstrated that intraperitoneal injections of a nitrogen mustard in mice at two different pH values resulted in different LD_{50} values, which could be accounted for on the basis of absorption rate differences resulting from pH–partition effects (3, 4). Other studies revealed that the route of injection may have either substantial effects on acute toxicity, as with procaine, or relatively small effects, as with isoniazid (5).

For the tetracycline class of antibiotics, maximum lipid solubility occurs at the isoelectric pH (pH_{iso}); for tetracycline itself, it occurs around pH 5.6 (6). The relative lipid solubility of tetracycline then decreases as the pH is raised or lowered on either side of the pH_{iso}. Thus, the objective of this study was to determine if there is a significant difference in acute toxicity values, as determined in mice receiving tetracycline injections, when the formulation pH is altered or when the route of parenteral administration is changed.

EXPERIMENTAL

Preparation of Tetracycline Solutions—Aqueous solutions of tetracycline hydrochloride[1] or solutions containing 60% (v/v) propylene glycol[2] were prepared immediately before injection. Both solutions contained 20 mg of tetracycline hydrochloride/ml. The propylene glycol–aqueous vehicle was necessary due to the minimal water solubility of tetracycline hydrochloride around the pH_{iso}, which resulted in marked precipitation when water alone was employed as the solvent. The pH values of the solution were adjusted using 1 *N* NaOH to 2.45, 8.20, and 11.00 in water and to 3.00, 5.50, and 9.00 in the propylene glycol–water solvent mixture.

LD_{50} Determinations—The LD_{50} was determined by the method of Litchfield and Wilcoxon (7) as suggested by the Food and Drug Administration (8). This method employs logarithmic probability graph paper and nomographs as the basis of the calculation.

For each test (*i.e.*, for each different pH of a given solvent system by a particular route of administration), 25 male albino mice[3], ~20 g, were randomly divided into five groups of five animals. The mice were injected with the appropriate dose of tetracycline hydrochloride on a milligrams per kilogram basis, employing the appropriate pH–solvent system. Doses for every one of the six pH–solvent systems tested were administered to each of the five groups in the following manner: one dose at the approximate LD_{50} value, previously estimated experimentally, and two doses above and two doses below this median point.

The exact doses were determined by converting the experimentally estimated doses to log scale and separating each dose by ap-

[1] Tetracycline hydrochloride, lot 174-010, donated by Lederle Laboratories, Division of American Cyanamid Co., Pearl River, N.Y.

[2] Propylene glycol, Ruger Chemical Co., Irvington, N.Y.

[3] Swiss-Webster male albino mice, Hilltop Farms, Scottdale, Pa.

Table I—Mean LD_{50} Values with 95% Confidence Limits[a] and Potency Ratios for Tetracycline Hydrochloride at Various pH Values and with Various Solvent Systems and Routes of Parenteral Administration in Mice

Vehicle and Route of Administration	LD_{50} and Confidence Limits, mg/kg			Potency Ratio and Confidence Limits		
	pH 2.45	pH 8.20	pH 11.00	pH 2.45 *versus* 8.20	pH 2.45 *versus* 11.00	pH 8.20 *versus* 11.00
Aqueous vehicle						
Intraperitoneal	485 (452–519)	366 (302–442)	415 (343–502)	1.33[b] (1.10–1.60)	1.17[c] (0.97–1.41)	1.13[c] (0.86–1.48)
Subcutaneous	673 (510–888)	651 (449–944)	657 (355–1215)	1.03[c] (0.65–1.63)	1.02[c] (0.52–1.99)	1.01[c] (0.49–2.07)
	pH 3.00	pH 5.50	pH 9.00	pH 3.00 *versus* 5.50	pH 3.00 *versus* 9.00	pH 5.50 *versus* 9.00
Propylene glycol–aqueous vehicle						
Intraperitoneal	475 (420–537)	327 (262–409)	377 (320–455)	1.45[b] (1.13–1.86)	1.26[b] (1.02–1.56)	1.15[c] (0.88–1.51)
Subcutaneous	681 (497–932)	623 (498–779)	652 (438–971)	1.09[c] (0.75–1.59)	1.04[c] (0.63–1.73)	1.05[c] (0.67–1.65)

[a] Shown within parentheses. [b] Statistically significant ($p < 0.05$). [c] Not statistically significant ($p > 0.05$).

proximately 0.1 log unit (8). Each test solution was administered, in a volume sufficient to provide the desired dose, by the intraperitoneal and subcutaneous routes to two separate sets of mice. The mice were then observed for 48 hr following injection, and the number of deaths for each of the five separate dosage groups was recorded for each set.

A control group of mice also was employed by administering the same vehicle including any additives, but devoid of tetracycline, on the appropriate volume per kilogram basis by the route of administration being employed.

RESULTS

The results of this study (Table I and Fig. 1) indicate that tetracycline hydrochloride, when administered to mice by the intraperitoneal route, produced greater toxicity in the pH_{iso} region (approximately pH 5.5) of tetracycline. The data suggest a trend toward decreasing toxicity as the pH is decreased or increased from the pH_{iso} value. The results of a potency ratio test[4] performed on the data (Table I) indicated that the LD_{50} values for pH 2.45 and 3.00 were significantly higher than the LD_{50} values at the pH_{iso}. However, as Table I shows, for those pH values above the pH_{iso}, statistically significant differences for LD_{50} values were not shown by the potency ratio test. This observation is probably due to the greater variability in the amounts of tetracycline required to produce death in the test animals at these higher pH regions.

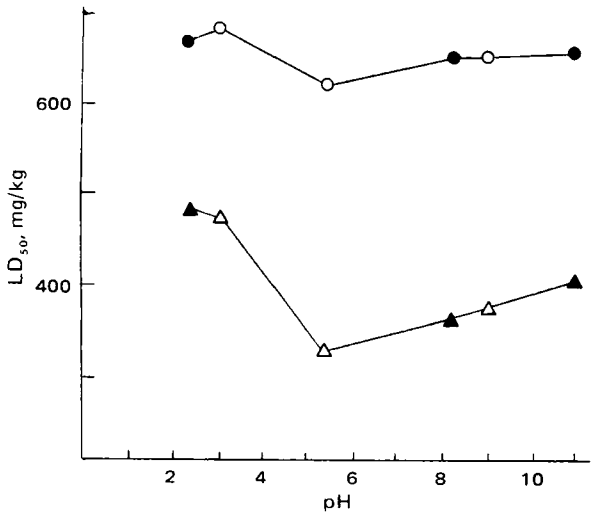

Figure 1—*Comparison of LD_{50} values for tetracycline hydrochloride at different pH values. Key: ●, subcutaneous route, water solvent; ○, subcutaneous route, water–propylene glycol solvent; ▲, intraperitoneal route, water solvent; and △, intraperitoneal route, water–propylene glycol solvent.*

[4] A test that compares the LD_{50} values obtained at separate pH levels and indicates if there is a statistically significant difference (5% level) between their toxicity values (7).

Figure 1 and Table I also show the results obtained when tetracycline hydrochloride was administered by the subcutaneous route. The general pattern was similar to the trend observed for the intraperitoneal route, with the greatest apparent toxicity occurring near the pH_{iso}. However, the magnitude of change was not as great, and the potency ratio test failed to demonstrate statistical significance at the 5% level.

The control group of mice, which received only drug-free solutions, showed no toxicity at any pH value by either route of administration.

DISCUSSION

The results of this study demonstrate that significant alterations in the acute toxicity characteristics of parenterally administered tetracycline hydrochloride may occur when either formulation pH or route of administration varies. Changing the pH of a tetracycline solution produced significant changes in the LD_{50} values when the drug was administered by the intraperitoneal route of administration in mice. When tetracycline was administered by the subcutaneous route, a similar trend of increased toxicity near the pH_{iso} region was also observed, but the differences were not statistically significant ($p > 0.05$). This finding could be attributed to the much slower absorption rates generally associated with the subcutaneous route, particularly in comparison with the intraperitoneal route (9).

Since the tetracycline moiety forms a zwitterionic species (6), the pH values selected were such that one experimental value would result in a tetracycline solution with a pH close to the maximum concentration of the zwitterionic form of the drug. The other pH values selected were far enough on either side of the pH_{iso} to represent a relatively high cationic species (pH 2.45–3.00) or a relatively high anionic species (pH 8.20–11.00) of the drug.

The results obtained, using the intraperitoneal route of administration, indicate that maximum zwitterionic species concentration results in the greatest toxicity for this compound. This finding appears to be due to the fact that when tetracycline hydrochloride exists as a zwitterion, it is at its pH of greatest lipid solubility (4). Since increased lipid solubility can generally increase the rate of drug absorption (10), it would follow that the zwitterionic pH produces greater toxicity since the drug is more rapidly absorbed when administered in this manner. For pH values above and below the pH_{iso}, tetracycline is in a less lipid-soluble form than for the zwitterionic pH region; therefore, it would tend to be less rapidly absorbed.

When attempts were made to maintain the pH of the tetracycline solutions employed in this study in the vicinity of the pH_{iso}

(5.5), a precipitate occurred immediately in a solvent system composed of water alone. Precipitate formation was finally avoided by the use of a solvent mixture of 60% propylene glycol in water. To check for the effect of solvent system throughout the test pH range, the propylene glycol–water mixture was used again in the cationic and anionic pH ranges. This evaluation as to possible solvent effects that might alter LD_{50} determinations revealed that the solvent mixture, as employed in this study, had little or no apparent influence on the absorption, as reflected by the toxicity data. Therefore, the data obtained using the propylene glycol–water solution could be pooled with the aqueous data to yield results indicative of alterations in the toxicological characteristics of tetracycline.

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[x] To whom inquiries should be directed.

Antileukemic and Other Constituents of *Tithonia tagitiflora* Desf.

RAGHWENDRA PAL, D. K. KULSHRESHTHA, and R. P. RASTOGI [x]

Abstract □ Phytochemical investigation of *Tithonia tagitiflora* has led to isolation of six new germacranolides, tagitinins A, B, C, D, E, and F, β-sitosterol, and its β-D-glucoside. Among these, tagitinin F possesses antileukemic activity.

Keyphrases □ *Tithonia tagitiflora*—whole plant alcoholic extract, isolation of six new germacranolides, β-sitosterol, and its β-D-glucoside, screened for antileukemic activity □ Germacranolides—six new tagitinins isolated from whole plant extract of *Tithonia tagitiflora*, screened for antileukemic activity □ β-Sitosterol and its β-D-glucoside—isolated from whole plant extract of *Tithonia tagitiflora* □ Antileukemic agents, potential—constituents isolated from whole plant extract of *Tithonia tagitiflora* screened

Tithonia tagitiflora Desf. (Compositae) is an American plant which has become naturalized in the Khandala region. Only one species of this genus, namely *T. tubaeformis*, has undergone chemical investigation, resulting in the characterization of a new flavone, tithonine (1), and a novel germacranolide, orizabin (2).

In the screening program for tumor inhibitors from plant sources (3), the alcoholic extract of *T. tagitiflora* showed significant activity against P-388 lymphocytic leukemia (PS). The results of the subsequent studies leading to the isolation of the various constituents together with their biological data are described here[1].

[1] The plant material was identified as *Tithonia tagitiflora* Desf. (Compositae) by Mr. B. N. Mehrotra, Section of Botany, Central Drug Research Institute, Lucknow, India. A voucher (preserved) specimen (No. 2499), representing material collected for this investigation, is available for inspection at the Herbarium of the Institute.

EXPERIMENTAL

The reported melting points are uncorrected. Spots on TLC were visualized with 1% ceric sulfate in 2 *N* H_2SO_4 using silica gel plates and with alkaline potassium permanganate using neutral alumina plates.

Isolation of Constituents—The alcoholic extract of the dried plant material (11.0 kg) was extracted with benzene. The residual portion was partitioned with 1-butanol–water. The benzene-soluble material was defatted with *n*-hexane, and the insoluble fraction was decolorized with charcoal in ethanol, which yielded a light-green viscous mass (165 g). This residue showed eight spots on TLC which were designated as α (R_f 0.55 in ethyl acetate saturated with water + 10% methanol), A [R_f 0.44 in chloroform–ethyl acetate (1:1)], B, C, D, E, F, and G [R_f 0.22, 0.27, 0.33, 0.40, 0.47, and 0.58, respectively, in benzene–ethyl acetate (2:1)] on neutral alumina plates. A portion of the residue (68 g) was subjected to a gross fractionation over silica gel (1 kg) as shown in Table I.

The residue from fractions 3–5, on repeated crystallization from methanol, gave substance G, mp 136° (4.0 g).

The residue from fraction 7 (7.7 g) was rechromatographed over neutral alumina (activity of 2.5, 350 g). The residue (1.5 g) from the benzene–ethyl acetate (9:1) eluate crystallized from hexane–benzene, mp 128–130° (tagitinin F, 0.75 g), and the benzene–ethyl acetate (7:3) eluate (1.44 g) crystallized in a similar manner to yield tagitinin E, mp 208–210°, 0.50 g.

Fraction 9 (Table I, 4.2 g) was rechromatographed over neutral alumina. The benzene–ethyl acetate (7:3) eluate (1.11 g), on repeated crystallization from benzene–hexane, gave tagitinin D, mp 138–140° (0.45 g). The residue from the benzene–ethyl acetate (3:2) eluate (0.80 g) was rechromatographed over silica gel impregnated with silver nitrate (12%), and the benzene–ethyl acetate (3:2) eluate yielded pure tagitinin C (0.33 g) as colorless powder.

Rechromatography of fraction 10 (Table I, 5.0 g) over neutral alumina yielded a benzene–ethyl acetate (1:1) eluate (1.15 g), which crystallized from acetone–isopropyl ether, mp 125–126° (tagitinin