safely carried out in the presence of crataegus extract and other alkaloids such as papaverine, strychnine, morphine, and atropine.

Although the proposed reactions are specific for quinine and quinidine, a TLC procedure is necessary for cinchona preparations to separate quinine and quinidine and to eliminate other interfering constituents. The four main cinchona alkaloids showed good separation; the $R_f$ values of quinine, quinidine, cinchonine, and cinchonidine were 0.18, 0.44, 0.55, and 0.41, respectively.

Ammonium hydroxide was not capable of releasing quinine base from the dipyrone–quinine salt, while sodium hydroxide solution was satisfactory.

The proposed methods also were applied successfully to the recovery of quinine and quinidine from blood and urine with reproducible results. With blood, because of the small concentration of the alkaloids present, only the more sensitive erythroquinine method was adopted.

Good recoveries at different concentrations and spiking and reasonable standard deviations were obtained with dosage forms and biological fluids by the erythroquinine and thalleioquin methods (Tables I–III).

To obtain protein-free aqueous filtrates from blood and urine, the samples were digested with hydrochloric acid to liberate strongly conjugated alkaloids. Hydrochloric acid digestion was preferable to other procedures.

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# High-Pressure Liquid Chromatographic Determination of Tetracyclines in Urine


**J. P. SHARMA [*], G. D. KORITZ [*], E. G. PERKINS [‡], and R. F. BEVILL [*x]**



**Abstract** □ The quantitation of oxytetracycline, tetracycline, and chlortetracycline was accomplished by high-pressure liquid chromatography using an anion-exchange column. The tetracyclines were extracted from urine as their calcium complexes. Concentrations as low as 12 μg of oxytetracycline/ml and 4 μg of tetracycline and chlortetracycline/ml were quantitated accurately. The relative standard deviation of the method varied from 0 to 5%.



**Keyphrases** □ Oxytetracycline—high-pressure liquid chromatographic analysis, urine □ Tetracycline—high-pressure liquid chromatographic analysis, urine □ Chlortetracycline—high-pressure liquid chromatographic analysis, urine □ High-pressure liquid chromatography—analyses, oxytetracycline, tetracycline, and chlortetracycline in urine □ Antibacterials—oxytetracycline, tetracycline, and chlortetracycline, high-pressure liquid chromatographic analyses, urine


To establish the urinary excretion rate of tetracyclines in the urine of cattle, sheep, and swine following intravenous drug administration, an accurate method for the quantitation of oxytetracycline, tetracycline, and chlortetracycline was required. TLC (1–5) and paper chromatographic (6–9) methods were laborious and lacked sufficient sensitivity and accuracy. One GLC method (10) required the formation of trimethylsilyl derivatives and sometimes resulted in the formation of tetracycline degradation products. None of the previously reported fluorometric methods (11–14) could be used due to large and variable amounts of fluorescent material in urine obtained from untreated control animals.

Several high-pressure liquid chromatographic (HPLC) methods for the separation and determination of tetracyclines have been reported. A low efficiency column packing was used for the separation of tetracyclines, but the method lacked the sensitivity required for analysis of biological samples (15). The experimental conditions required for the qualitative separation of several tetracyclines were discussed (16), but the quantitative utility of this method was not reported. Several reports (17, 18) did not include oxytetracycline. The purpose of this study was

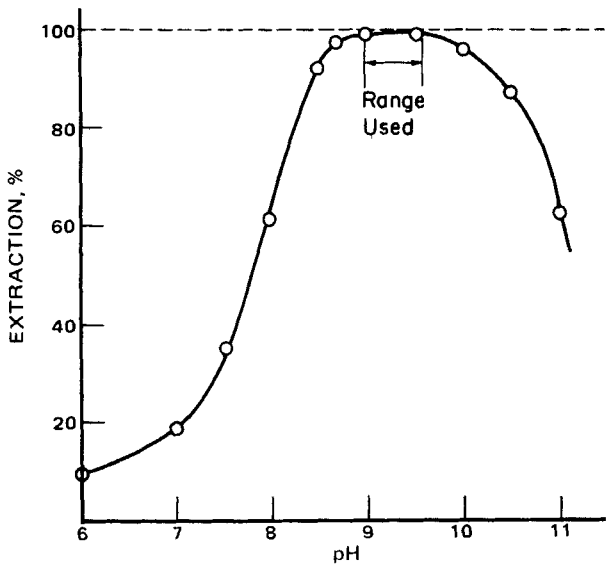


**Figure 1**—*Effect of pH on the extraction of tetracycline–calcium complexes from cattle urine.*

to develop an HPLC method for the quantitation of tetracyclines in urine.

## EXPERIMENTAL

**Apparatus**—An HPLC unit[1] equipped with a variable wavelength UV detector[2] was used. The UV spectra of the tetracyclines were recorded with a double-beam spectrophotometer[3].

**Reagents and Materials**—Mobile Phase A was 0.005 *M* ethylenediaminetetraacetic acid[4] (disodium salt) (I), 0.05 *M* NaCl[5], and 5% methanol[6] in distilled water; Phase B was 0.005 *M* I, 0.05 *M* NaCl, and 30% methanol in distilled water. The distilled water was degassed prior to use, and the pH of each mobile phase was adjusted to 9.9 with ammonium hydroxide[7] solution.

A 1.0 *N* solution of hydrochloric acid[7] was used for back-extraction of tetracyclines. A trichloroacetic acid[8]–calcium chloride[8] reagent was prepared by dissolving 40.0 g of trichloroacetic acid and 4.0 g of calcium chloride dihydrate in 100 ml of distilled water. The sodium hydroxide solution[8] contained 9 g of sodium hydroxide/100 ml of water. Barbital sodium[8] contained 10.3 g/100 ml of distilled water. Fresh solutions were prepared every 2 weeks. Pure standards of oxytetracycline[9], tetracycline[10], and chlortetracycline[10] as the hydrochlorides were dried *in vacuo* prior to use.

Columns were dry packed with anion-exchange material[11] after conditioning in a 0.02 *M* solution of ethylenediaminetetraacetic acid.

**Chromatographic Procedures**—Isocratic solvent elutions with Mobile Phase A (oxytetracycline) or B (tetracycline and chlortetracycline) were used for tetracycline quantitation. Stock standards were prepared by adding 50 mg of the tetracycline to be quantitated (sample drug) and an internal standard (a second tetracycline) to separate 100-ml volumetric flasks containing distilled water and 0.5 ml of 1.0 *N* hydrochloric acid. Working standards were prepared by combining aliquots of each stock standard in a 25-ml flask and diluting with distilled water.

Duplicate 3-ml aliquots of urine were placed in 20-ml screw-capped tubes and spiked with 1 ml of the working standard. The trichloroacetic acid–calcium chloride reagent (1 ml) was added to each tube, which was then agitated (2 min) and centrifuged (2000 rpm for 10 min). The supernates (4.5 ml) were transferred to tubes containing 1.0 ml of both sodium hydroxide and barbital sodium solutions.

The contents of the tubes were partitioned with 5.0 ml of ethyl acetate and centrifuged, and aliquots (8 μl) of the upper layer were injected on the column. An average response factor was calculated from the ratio of the peak heights produced by the sample drug and the internal standard as follows:

$$\text{response factor } (f) = \frac{H_s}{H_{std}} \times \frac{W_{std}}{W_s} \qquad \text{(Eq. 1)}$$

where $H_s/H_{std}$ is the ratio of the peak heights of the sample drug to the internal standard, and $W_{std}/W_s$ is the weight ratio of the internal standard to the sample drug.

Urine samples containing variable amounts of the sample drug were spiked with known amounts of the internal standard and prepared for injection as previously described. Following injection of the urine extracts, the amount of sample drug was calculated using:

$$\text{amount} = \frac{R \times W'_{std}}{f} \qquad \text{(Eq. 2)}$$

where $R$ is the ratio of peak heights of the unknown amount of sample drug to the known amount of internal standard ($W'_{std}$).

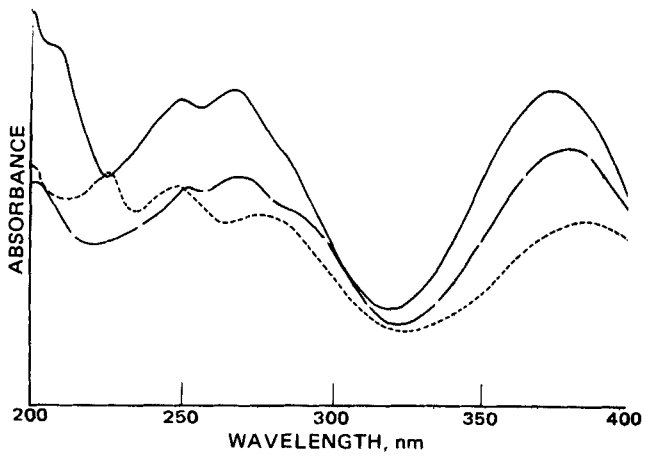


**Figure 2**—*UV absorption spectrum of tetracycline (– –), oxytetracycline (—), and chlortetracycline (- - -) in Mobile Phase A.*

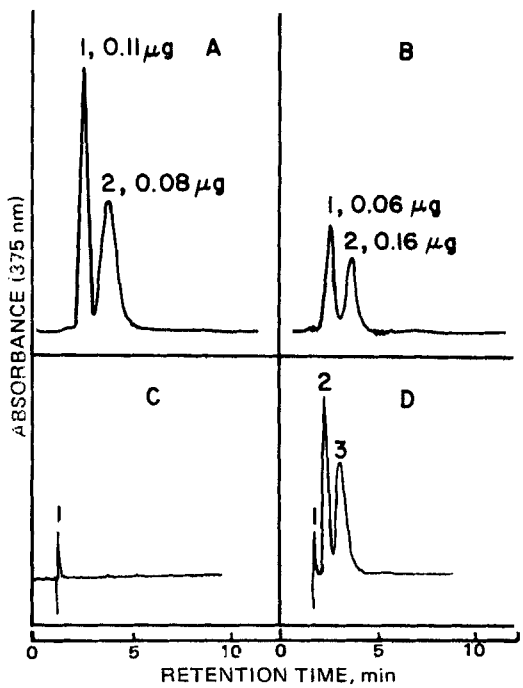


**Figure 3**—*(A) Elution of oxytetracycline (1) and tetracycline (2) standards with Mobile Phase A. (B) Elution of tetracycline (1) and chlortetracycline (2) standards with Mobile Phase B. (C) Chromatogram of swine urine extract blank with Mobile Phase A showing only the solvent peak (1). (D) Chromatogram of the extract of swine urine spiked with 50 μg of oxytetracycline/ml (2) and 50 μg of tetracycline/ml (3) with Mobile Phase A. Peak 1 is the solvent peak.*

[1] Model 1220, Perkin-Elmer Corp., Norwalk, Conn.
[2] Model LC-55, Perkin-Elmer Corp., Norwalk, Conn.
[3] Model 25, Beckman Instruments, Fullerton, Calif.
[4] Reagent grade, Hach Chemical Co., Ames, Iowa.
[5] Certified ACS, Fisher Scientific Co.
[6] Spectroscopic grade, Burdick and Jackson, Muskegan, Mich.
[7] Analytical reagent, du Pont, Wilmington, Del.
[8] Analytical reagent, Mallinckrodt, St. Louis, Mo.
[9] Sigma Chemical Co., St. Louis, Mo.
[10] Lederle Laboratories, Division of American Cyanamid, Pearl River, N.Y.
[11] Ion-X-SA, Perkin-Elmer Corp., Norwalk, Conn.

**Table I—Determination of Tetracyclines in Swine, Sheep, and Cattle Urine**

| Tetracycline | Average Response Factor | Amount Spiked, μg/ml | Amount Recovered, μg/ml | Recovery, % (Average Recovery, %) | ± RSD[a], % |
|---|---|---|---|---|---|
| | | | Swine | | |
| Oxytetracycline | 1.305 (±0.007) | 12.8 | 12.7 | 99.2 | 2.3 |
| | | 30.0 | 30.5 | 107.6 | 2.2 |
| | | 50.0 | 51.5 | 103.0 | 1.3 |
| | | 70.0 | 68.5 | 97.8 | 1.0 |
| | | | | (101.9) | |
| Tetracycline | 0.964 (±0.006) | 4.3 | 4.4 | 102.3 | 0.5 |
| | | 37.0 | 36.5 | 98.6 | 1.9 |
| | | 62.0 | 60.0 | 96.7 | 0.0 |
| | | 87.0 | 89.5 | 102.8 | 0.8 |
| | | | | (100.1) | |
| Chlortetracycline | 0.263 (±0.019) | 7.0 | 7.3 | 104.2 | 2.3 |
| | | 44.0 | 49.0 | 111.3 | 2.8 |
| | | 73.0 | 73.0 | 100.0 | 1.9 |
| | | 103.0 | 104.0 | 100.9 | 2.7 |
| | | | | (104.1) | |
| | | | Sheep | | |
| Oxytetracycline | 0.999 (±0.015) | 17.0 | 17.2 | 101.1 | 2.2 |
| | | 66.0 | 61.0 | 92.4 | 5.0 |
| | | 93.0 | 91.0 | 97.8 | 2.3 |
| | | | | (97.1) | |
| Tetracycline | 0.997 (±0.017) | 5.7 | 5.8 | 101.7 | 5.4 |
| | | 35.0 | 36.5 | 104.2 | 4.5 |
| | | 77.0 | 77.5 | 100.6 | 3.8 |
| | | 101.0 | 102.0 | 100.9 | 2.0 |
| | | | | (101.8) | |
| Chlortetracycline | 0.249 (±0.015) | 9.0 | 8.6 | 95.5 | 0.3 |
| | | 49.0 | 51.0 | 104.0 | 2.7 |
| | | 59.0 | 64.0 | 108.4 | 5.4 |
| | | 74.0 | 71.0 | 95.9 | 1.9 |
| | | | | (100.9) | |
| | | | Cattle | | |
| Oxytetracycline | 1.315 (±0.007) | 20.0 | 19.4 | 97.0 | 0.5 |
| | | 66.0 | 65.5 | 99.2 | 1.0 |
| | | 133.0 | 134.5 | 101.1 | 0.5 |
| | | | | (99.1) | |
| Tetracycline | 0.856 (±0.003) | 7.5 | 7.8 | 104.0 | 1.2 |
| | | 39.0 | 40.0 | 102.5 | 2.0 |
| | | 72.0 | 72.5 | 100.6 | 0.9 |
| | | | | (102.3) | |
| Chlortetracycline | 0.298 (±0.014) | 10.0 | 9.3 | 93.0 | 0.3 |
| | | 49.0 | 46.5 | 94.8 | 1.5 |
| | | 59.0 | 63.0 | 106.7 | 4.4 |
| | | 74.0 | 74.0 | 100.0 | 2.8 |
| | | | | (98.6) | |

[a] Duplicate samples were analyzed at each concentration.

## RESULTS AND DISCUSSION

Tetracyclines form extractable complexes with $Ca^{2+}$ when combined in a weakly basic medium (11, 19). A series of aqueous samples containing 30 μg of tetracycline/ml were adjusted to different pH values, from 6 to 11, with hydrochloric acid or sodium hydroxide and extracted with equal volumes of ethyl acetate. The extraction efficiency was determined by comparing the peak heights obtained after equal volumes of the aqueous and organic phases from each sample were injected. The extraction profile is presented in Fig. 1. Optimum extraction of tetracycline was obtained at pH 8.5–10 and was the same for other tetracyclines.

Extraction of urine samples was preceded by a deproteinization step using 40% trichloroacetic acid. During this process, 10–20% of the tetracyclines present in the urine sample was lost. Greater losses occurred during the deproteinization of cattle urine, whereas the losses associated with the deproteinization of swine urine were lower. Following deproteinization, the urine was adjusted to pH 9.0–9.5 with sodium hydroxide and 0.5 *M* barbital sodium. The extraction of oxytetracycline from the urine of cattle, sheep, and swine varied from 65 to 72%, but greater extraction efficiencies (98–99%) were realized with tetracycline and chlortetracycline. The extraction of all three drugs was reproducible, and no deterioration of the extracted calcium complexes was noted during the first 10 hr following their partition into ethyl acetate.

UV spectroscopy at 254 and 280 nm was used previously for the detection of tetracyclines (15–17). In the present study, ethyl acetate (UV cutoff 260 nm) produced a pronounced solvent peak at 254 nm which interfered with the determination of oxytetracycline. Similar complications were encountered when monitoring was performed at 280 nm as a result of the extraction of UV-absorbing components in urine simultaneously eluting with the tetracyclines. To circumvent these problems, 375 nm was selected on the basis of the UV spectra presented in Fig. 2.

Two solvent systems were required to reduce the elution time of each tetracycline and to provide for separation of the sample drug and the internal standard. Oxytetracycline, tetracycline, and chlortetracycline were separated with Mobile Phase A, but the retention of chlortetracycline was prolonged. Chlortetracycline and tetracycline were separated and the chlortetracycline retention time was greatly reduced with Mobile Phase B, but oxytetracycline eluted with the solvent front.

On the basis of these findings, oxytetracycline was quantitated in Mobile Phase A using tetracycline as an internal standard (Fig. 3A). Mobile Phase B was used for the quantitation of tetracycline and chlortetracycline with either of the two drugs serving as an internal standard (Fig. 3B). The solvent peak, which appeared when urine extracts were injected (Fig. 3C), was well separated from sample peaks (Fig. 3D) irrespective of the use of Mobile Phase A or B. The results of the determination of the three tetracyclines in swine, sheep, and cattle urine are presented in Table I. The relative standard deviation of the method ranged from 0 to 5%.

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# Drug Interactions with Isoniazid Metabolism in Rats

**BARRY H. THOMAS ˣ and GNANAPRAKASAM SOLOMONRAJ**

**Abstract** □ $^{14}$C-Isoniazid (20 mg/kg po or iv) was administered alone or in combination with aspirin (100 mg/kg po), rifampin (30 mg/kg po), ethambutol (100 mg/kg po), or ethanol (3 g/kg po) to rats. In another experiment, phenobarbital sodium (40 mg/kg/day ip) was administered for 3 days prior to isoniazid. Aspirin and ethanol retarded the rate of isoniazid absorption from the GI tract. None of the drugs significantly altered the $^{14}$C-elimination rate from the blood over the first 4 hr. A tissue distribution study showed that changes in the blood levels produced by ethanol were reflected in the other tissues. When isoniazid was given intravenously, ethanol increased the amount of carbon-14 excreted in urine up to 24 hr after dosing; no other changes were observed in the total carbon-14 recovered in urine. Aspirin inhibited the conjugation of isonicotinic acid with glycine. Ethanol increased *N*-acetylisoniazid excretion and decreased isonicotinic acid excretion. None of the other treatments had more than a slight effect on isoniazid metabolism. Acute doses of isoniazid failed to produce any signs of hepatotoxicity, as judged by measurement of serum transaminase levels. The data do not suggest that any of the drugs studied are likely to potentiate the hepatotoxicity of isoniazid when administered acutely. Isoniazid metabolism in rats differed quantitatively from that reported for humans.

**Keyphrases** □ Isoniazid—absorption, tissue distribution, excretion, and metabolism, effect of aspirin, rifampin, ethambutol, ethanol, and phenobarbital, radiochemical analysis, rats □ Absorption—isoniazid, effect of various drugs, radiochemical analysis, rats □ Distribution, tissue—isoniazid, effect of various drugs, radiochemical analysis, rats □ Excretion—isoniazid, effect of various drugs, radiochemical analysis, rats □ Metabolism—isoniazid, effect of various drugs, radiochemical analysis, rats □ Radiochemistry—analysis, isoniazid, effect of various drugs on absorption, tissue distribution, excretion, and metabolism, rats □ Interactions, drug—isoniazid, absorption, tissue distribution, excretion, and metabolism, effect of aspirin, rifampin, ethambutol, ethanol, and phenobarbital, radiochemical analysis, rats □ Antibacterials, tuberculostatic—isoniazid, absorption, tissue distribution, excretion, and metabolism, effect of various drugs, rats

Interest in the metabolism and toxicity of isoniazid was aroused by the death of two patients and the development of clinical hepatotoxicity in 17 others in 1970 following an isoniazid chemoprophylactic program (1). Furthermore, the antituberculous drug rifampin increased the incidence of hepatotoxicity in humans when given with isoniazid (2). This same association was observed in rats (3).

Recently, it was shown that metabolism by the microsomal mixed function oxidase system is responsible for isoniazid hepatotoxicity (4). It was suggested that it is necessary to acetylate isoniazid before it can be a substrate for the toxication pathway. The purposes of this study were to screen drugs likely to be taken by tuberculous patients and to observe their influence on isoniazid metabolism as a clue to possible toxic drug interactions.

## EXPERIMENTAL

[$^{14}$C-Carboxyl]-isoniazid[1] had a specific activity of 11.3 mCi/mmole and a radiochemical purity of 96–97%, as determined in two paper chromatography and two TLC systems. Aspirin[2], ethambutol[3], rifampin[4], phenobarbital sodium[5], and absolute ethanol[6] were obtained commercially.

Male Wistar rats[7], 170–200 g, were deprived of food but not water for 16 hr prior to dosing and were housed in metabolism cages[8] that separated urine and feces. In most experiments, $^{14}$C-isoniazid was given orally at a dose of 20 mg/kg in 10 ml of water/kg. In one experiment, $^{14}$C-isoniazid was given intravenously into the saphenous vein at a dose of 20 mg/kg in 1.0 ml/kg. The dose of carbon-14 in all experiments was 25 μCi/kg.

Aspirin (100 mg/kg) in 0.25% gum tragacanth, ethambutol (100 mg/kg), rifampin (30 mg/kg), and ethanol (3 g/kg) were given orally immediately prior to the dose of isoniazid in 10 ml of water/kg. Phenobarbital sodium, 40 mg/kg/day ip, was administered for 3 days prior to isoniazid. Control animals were dosed similarly with saline for 3 days.

Duplicate 10-μl blood samples were collected from the tail at 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, and 12 hr after dosing using calibrated capillary tubes. The total carbon-14 in the samples was determined by digestion and

[1] Amersham/Searle, Oakville, Ontario, Canada.
[2] J. T. Baker Chemical Co., Phillipsburg, N.J.
[3] Myambutol, Cyanamid of Canada, Montreal, Quebec, Canada.
[4] Rimactane, Ciba-Geigy Canada Ltd., Dorval, Quebec, Canada.
[5] B.D.H. Canada Ltd., Toronto, Ontario, Canada.
[6] Consolidated Alcohols Ltd., Toronto, Ontario, Canada.
[7] Woodlyn Farms, Guelph, Ontario, Canada.
[8] Model 4-640-000, Acme Research Products, Cincinnati, Ohio.