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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF TETRACYCLINES IN URINE AND PLASMA

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SUMMARY

The high-performance liquid chromatographic separation and quantitative analysis of oxytetracycline, tetracycline, and chlortetracycline from urine and plasma were effected on an octadecylsilane reversed-phase packing. The calcium complexes of the tetracyclines were extracted from urine and plasma with ethyl acetate and then re-extracted into hydrochloric acid. Following the injection of the hydrochloric acid extracts onto the column, the individual tetracyclines were eluted isocratically and quantitated spectrophotometrically. Concentrations of less than 1 μg/ml in urine and 1.5 μg/ml in plasma were quantitatively determined with a relative standard deviation of less than 5%.

INTRODUCTION

A rapid, accurate and sensitive method for the analysis of tetracyclines was needed to study the concentrations of these antibiotics in the plasma and urine of cattle sheep, and swine. Previously reported[1–12] chromatographic methods were laborious and often lacked the sensitivity required to detect tetracyclines in biological materials. Fluorometric methods[13–16] were unsuited to the analysis of urine, as large and variable amounts of endogenous fluorescent materials interfered with drug analysis. Several high-performance liquid chromatographic (HPLC) methods[17–21] had been applied to the analysis of tetracyclines in pharmaceutical preparations. These methods were not satisfactory for the analysis of plasma and urine containing tetracyclines because they failed to separate the drugs from other ultraviolet (UV) absorbing components in these fluids. The use of an HPLC method for the analysis of tetra-

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cyclines in the urine of food-producing animals which utilized an anion-exchange packing to effect quantitation of the drugs has been reported[22]. The method was useful but lacked the sensitivity required to study the lower concentrations of tetracyclines found in plasma and urine during the final stages of drug elimination. An improved HPLC system utilizing a μBondapak/C_{18} reversed-phase packing and isocratic solvent elution system which provides increased sensitivity is described in this paper.

EXPERIMENTAL

Apparatus

A liquid chromatograph (Perkin-Elmer, Model 1220) equipped with a 30 cm × 4.5 mm I.D. column packed with octadecylsilane bonded phase material (μBondapak/C_{18}, Waters Assoc., Milford, Mass., U.S.A.) was used to effect separations. Column eluants were monitored with a variable-wavelength UV-visible detector containing an 8-μl flow cell (Perkin-Elmer, Model LC-55). The detector output was recorded on a variable-span potentiometric recorder (Linear Instruments). The UV spectra of tetracyclines in the mobile phases were recorded with a double-beam spectrophotometer (Beckman, Model 25).

Reagents and materials

Mobile phases. The mobile phases used were: (A) 0.01 *M* sodium dihydrogen phosphate monohydrate (Analytical Reagent grade, Mallinckrodt, St. Louis, Mo., U.S.A.), 30% acetonitrile in distilled water, pH 2.4 and (B) 0.01 *M* sodium dihydrogen phosphate monohydrate, 40% acetonitrile in water, pH 2.4.

Distilled water was degassed and the pH of each mobile phase was adjusted with dilute nitric acid. Glass-distilled non-spectrograde acetonitrile (Burdick and Jackson Labs., Muskegon, Mich., U.S.A.) was used as received.

Extraction reagents. For the preparation of trichloroacetic acid (TCA)–calcium chloride reagent 40 g TCA and 4 g calcium chloride dihydrate (Mallinckrodt) were dissolved in 100 ml distilled water. Calcium chloride (4% solution), sodium hydroxide (9% solution), sodium barbital (0.5 *M* and 0.8 *M*, fresh solutions prepared every two weeks), and ethyl acetate were all analytical-reagent grade (Mallinckrodt). All acids (perchloric, nitric, hydrochloric, and sulfuric) were analytical-reagent grade materials.

Pure standards of oxytetracycline (Sigma, St. Louis, Mo., U.S.A.), tetracycline, and chlortetracycline (Lederle Labs., American Cyanamid Co., Pearl River, N.Y., U.S.A.) as their hydrochlorides were dried *in vacuo* prior to use. Samples (6–10 mg) were placed in dry 100-ml volumetric flasks and dissolved in distilled water just prior to use. Aliquots of these stock standards were diluted to 25 ml and used as working standards to spike urine and plasma.

Chromatographic conditions

All chromatographic procedures were done at ambient temperature with a flow-rate of 1.0 ml/min at approx. 1100 p.s.i. Recordings were made at a chart speed of 8 in./h and a recorder setting of 0.019 a.u.f.s. Injection volumes of 8 μl were introduced onto the column through a septum under stop-flow conditions. A wavelength of 355 nm was used to monitor column eluants.

Extraction procedures and preparation of standard curves

Urine. Two milliliters of urine and one milliliter of a tetracycline working standard were added to 20-ml glass tubes equipped with PTFE-lined screw caps. The resulting "spiked" urine contained 0.5–10 μg of a tetracycline/ml. TCA–calcium chloride reagent (1 ml) was added to the tubes, which were subsequently agitated for 1 min and centrifuged for 10 min at 2000 rpm. The clear supernatant was decanted into tubes containing 1.0 ml of both sodium hydroxide solution (9.0%) and sodium barbital solution (0.5 *M*). After 5 ml of ethyl acetate had been added, the tubes were agitated for 2 min and then centrifuged for 5 min at 2000 rpm. Four milliliters of the ethyl acetate were transferred from each tube to tubes containing 1.0 ml of 0.5 *N* perchloric acid. In the case of oxytetracycline, two 5.0-ml extractions with ethyl acetate were performed and 9.0 ml of the extract were transferred to the tubes containing 1.0 ml of 0.5 *N* hydrochloric acid. The tube contents were agitated and centrifuged for 2–3 min. The ethyl acetate layer was discarded and 8 μl of the acid layer were injected on the column. Each analysis was done in duplicate and standard curves were prepared by plotting the concentration of antibiotic (μg/ml) *versus* average peak height.

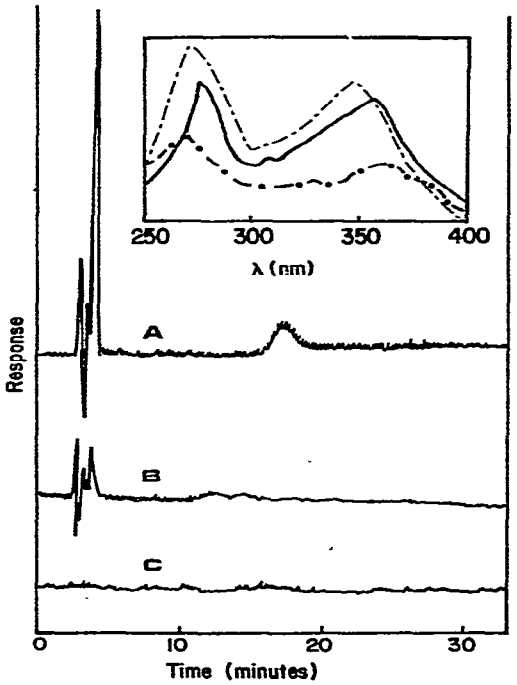
Plasma. Two milliliters of plasma were added to screw-capped tubes containing 1.0 ml of a tetracycline. One milliliter of both 4% calcium chloride and 0.8 *M* sodium barbital sclution were added to each tube. The remainder of the extraction procedure was conducted as previously described for urine.

RESULTS AND DISCUSSION

An octadecylsilane bonded phase packing was suitable for the separation and quantitation of nanogram quantities of tetracycline, oxytetracycline, and chlortetracycline in pure solution. However, column clogging and the simultaneous elution of normal urine components and tetracyclines occurred when plasma and urine samples "spiked" with tetracyclines were injected onto the packed column. To circumvent these problems, tetracyclines were selectively extracted with ethyl acetate prior to their injection onto the column. Maximum extraction efficiency was obtained when urine and plasma were buffered to pH 9.0–9.5 with sodium barbital and tetracyclines were converted to their calcium complexes[22]. Prior precipitation of proteins from urine increased the efficiency of tetracycline extraction. Precipitation proved unacceptable for the removal of proteins from plasma, as gel-like masses which interfered with the extraction process were formed when TCA was added to undiluted plasma. The extraction of tetracycline and chlortetracycline was essentially complete following a single partition with ethyl acetate, whereas two partition steps were required for efficient transfer of oxytetracycline to the solvent.

When tetracycline–calcium complexes are dissolved in acid, the complexes dissociate and the antibiotics are converted into their ammonium ions, which are highly soluble in aqueous phases. The addition of small volumes of hydrochloric acid to the urine and plasma extracts resulted in complete transfer of the tetracyclines from ethyl acetate to the acid. This partition produced a four- to ninefold concentration of the tetracyclines. This method of concentration did not cause tetracycline degradation and provided a simple alternative to the cumbersome process of evaporating the ethyl acetate under an inert atmosphere at controlled temperature.

When acid extracts of plasma and urine containing oxytetracycline, tetracycline and chlortetracycline were injected onto the octadecylsilane column packing and column eluants were monitored at 250 and 280 nm, the oxytetracycline peaks merged with those produced by UV-absorbing components extracted from urine (Figs. 1 and 2). The electronic absorption spectra of tetracyclines from 250–500 nm indicated that tetracyclines had an absorption band between 345 and 370 nm. Since the absorption maxima of oxytetracycline, tetracycline, and chlortetracycline were 350, 355, and 367 nm, respectively, a common wavelength of 355 was selected for their detection. At this wavelength, control urine and plasma extracts produced no detectable absorption peaks following their injection (Figs. 1 and 2).



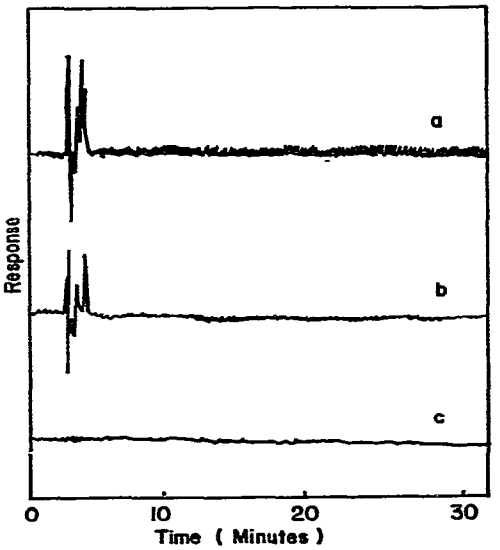


Fig. 1. Chromatograms of control swine urine extract with mobile phase B at (A) 254 nm, (B) 280 nm, and (C) 355 nm, and UV spectra of tetracyclines in mobile phase B. –·–·–·, oxytetracycline; ———, tetracycline; –●–●–, chlortetracycline.

Fig. 2. Chromatograms of control sheep plasma extract with mobile phase B at (a) 254 nm, (b) 280 nm, and (c) 355 nm.

Mobile phase B was used for all quantitative procedures involving tetracycline and chlortetracycline, as it decreased the retention times of the drugs and increased detection sensitivity (peak height) by factors of about 1.5 and 2.5, respectively. Mobile phase A proved more acceptable for the quantitation of oxytetracycline since the drug was eluted with the solvent front when mobile phase B was used (Fig. 3).

The results included in Table I reflect the precision of the extraction and chromatographic processes. The response factor (peak height/concentration) remained

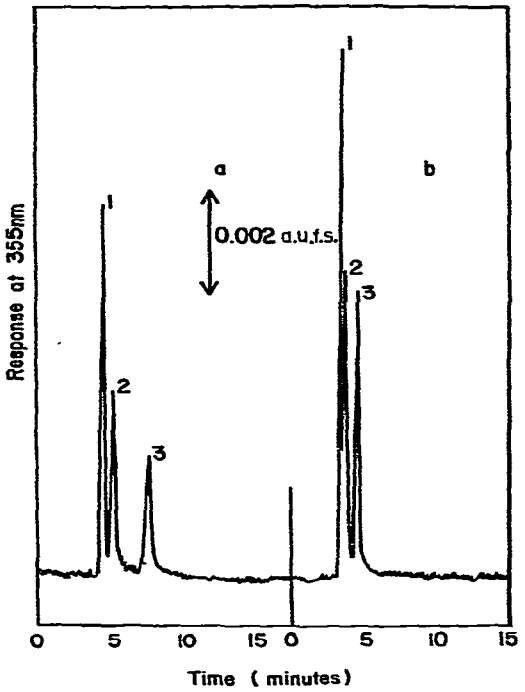


Fig. 3. Chromatograms of tetracyclines extracted from swine urine under the conditions described in the text with mobile phase A (a) and mobile phase B (b). Amounts spiked: (1) oxytetracycline, 20 μg/ml; (2) tetracycline, 10 μg/ml; (3) chlortetracycline, 21 μg/ml.

TABLE I

PRECISION OF THE OVERALL PROCEDURE FOR THE DETERMINATION OF TETRACYCLINE IN SWINE URINE

Amount of tetracycline spiked (μg/ml)	*Peak height* (mm)*	*Peak height/ concentration ratio*
0.64	5.0	7.80
1.20	9.0	7.50
1.30	9.5	7.30
2.58	19.0	7.36
3.80	28.0	7.37
3.90	29.0	7.43
7.76	57.0	7.34
12.95	101.0	7.80
	Av.	= 7.52
	R.S.D.	= 2.6%

* With mobile phase B.

constant over the range studied, but as shown in Fig. 4 the efficiency of tetracycline extraction from urine was species dependent. The extraction of a specific tetracycline from plasma was not species dependent but marked differences in the extraction of oxytetracycline, tetracycline, and chlortetracycline from plasma and urine were noted.

TABLE II

DETERMINATION OF TETRACYCLINES IN SWINE, SHEEP, AND CATTLE URINE

All the recovered values are averages of two separate determinations.

Tetracycline	*Swine*			*Sheep*			*Cattle*		
	Tetracycline added (μg/ml)	*Tetracycline recovered (μg/ml)*	*R.S.D. (%)*	*Tetracycline added (μg/ml)*	*Tetracycline recovered (μg/ml)*	*R.S.D. (%)*	*Tetracycline added (μg/ml)*	*Tetracycline recovered (μg/ml)*	*R.S.D. (%)*
Oxytetracycline	0.65	0.63	1.0	0.76	0.80	4.6	1.13	1.17	2.7
	1.23	1.32	2.5	1.72	1.77	4.0	3.08	2.95	2.3
	2.50	2.60	2.7	3.45	3.55	1.9	4.63	4.65	1.5
	3.70	3.67	0.9	8.63	8.80	1.6	10.80	11.0	2.5
Tetracycline	0.50	0.52	5.6	0.80	0.82	3.8	1.03	1.00	7.0
	3.88	3.87	0.9	1.85	1.75	0.9	2.07	2.10	3.3
	5.17	5.27	0.6	2.75	2.72	3.9	3.10	3.05	2.3
	7.76	7.75	0.9	5.54	5.67	1.8	6.21	6.20	2.3
Chlortetracycline	0.70	0.67	5.2	1.05	1.05	1.5	1.24	1.20	2.0
	1.44	1.50	4.6	2.10	2.02	1.7	2.48	2.50	2.8
	4.34	4.25	1.6	3.10	3.10	2.2	3.72	3.70	7.0
	8.70	8.70	2.4	6.25	6.15	0.6	7.43	7.30	2.5

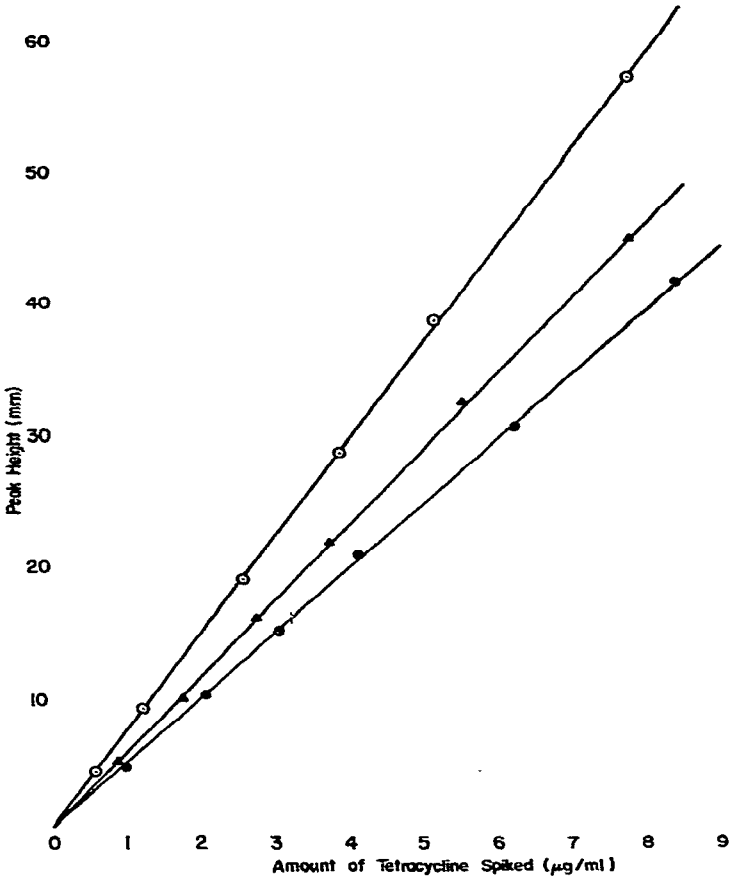


Fig. 4. Comparison of the extraction of tetracycline from swine, sheep, and cattle urine. ○, Swine; ▲, sheep; ●, cattle.

The negative intercepts and reduced slopes of the plasma curves presented in Fig. 5 indicate that the efficiency of tetracycline extraction from plasma was lower than that obtained with urine. The results obtained from the determinations of tetracyclines in swine, sheep, and cattle urine are presented in Tables II and III. The limits of assay sensitivity for oxytetracycline, tetracycline, and chlortetracycline in urine were 0.6, 0.5, and 0.7 μg/ml, respectively, whereas for plasma they were 3.0, 1.5, and 1.5 μg/ml, respectively. The activity of the column packing remained constant during extended use if, after each day's work, the inorganic ions were flushed from the column with a water–acetonitrile mixture and the manufacturers' recommendations regarding column maintenance were observed[23].

The effects of several other drugs on the extraction and quantitation of tetracyclines are presented in Table IV. When high concentrations of these compounds were dissolved in urine containing tetracycline, they did not interfere with the quantitation of the drug nor were they detected in column eluants. These observations sug-

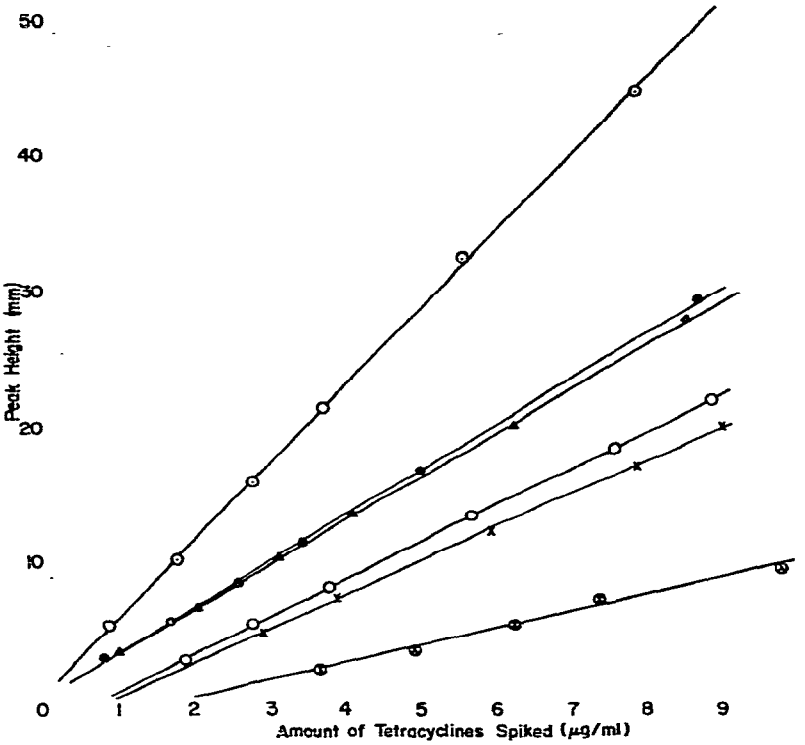


Fig. 5. Standard curves for the determination of tetracyclines in sheep urine and plasma. ⊙, Tetracycline in urine; ●, oxytetracycline in urine; ▲, chlortetracycline in urine; ○, tetracycline in plasma; ×, chlortetracycline in plasma; ⊗, oxytetracycline in plasma.

TABLE III

DETERMINATION OF TETRACYCLINES IN SHEEP PLASMA

All the recovered values are averages of two separate determinations.

Tetracycline	*Amount (μg/ml)*		*Relative standard deviation (%)*
	Added	*Recovered*	
Oxytetracycline	2.46	0.00	—
	3.70	3.65	4.0
	7.40	7.67	3.2
	9.86	9.47	5.6
Tetracycline	0.95	0.0	—
	1.89	1.90	3.0
	5.69	5.75	1.2
	7.58	7.6	2.8
Chlortetracycline	0.98	0.00	—
	2.00	2.10	2.0
	3.90	3.92	2.7
	7.85	7.80	5.3

TABLE IV

LIST OF COMPOUNDS TESTED FOR POSSIBLE INTERFERENCE IN THE DETECTION AND DETERMINATION OF TETRACYCLINES*

Abbreviations: NE = no effect; ME = minor effect; VME = very minor effect.

Compound	*Amount added (μg/ml)*	*Comment*
Sodium penicillin G	60	NE
Sulfathiazole	60	NE
Sulfamerazine	50	NE
Sulfachloropyridazine	55	NE
Sulfamethazine	60	NE
Sulfapyridine	60	NE
Sulfadiazine	60	NE
Sulfaguanidine	55	NE
Sulfadimethoxine	60	NE
Dihydrostreptomyc insulfate	50	NE
Neomycin	40	NE
Erythromycin	50	NE
Phosphate	60	ME
Oxalate	60	VME

* Tetracycline concentration = approx. 5–6 μg/ml.

gest that this method may be used to screen animal urine and plasma for the presence of tetracyclines and quantitatively determine them.

This method had two distinct advantages when compared to the method previously reported by Tsuji and Robertson[24], which employed gradient elution of tetracyclines from μBondapak/C_{18} columns. First, the isocratic elution conditions reduced the elapsed time between injections since the interval required to reverse the solvent gradient or otherwise establish the original starting conditions was eliminated. Secondly, the efficiency of the column was retained over an extended time period. The main advantages of the present method were the short analysis time (approx. 5 min for individual tetracyclines), isocratic elution conditions, high precision and accuracy, column stability, and selective detection of tetracyclines at concentrations less than 1 μg/ml.

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