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DETERMINATION OF TETRACYCLINE ANTIBIOTICS IN TISSUES AND BLOOD SERUM OF CATTLE AND SWINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Tissue homogenates and blood serum were acidified with hydrochloric acid and deproteinized with acetonitrile. Tetracyclines were partitioned into water and concentrated by solid-phase absorption on the analytical column from 0.01 M phosphoric acid–methanol (80:20). Tetracyclines were eluted with an acetonitrile gradient. An all-organic polymeric column (Polymer Labs. PLRP-S) was used. Similar results were obtained on a bonded reversed-phase column after addition of tetramethylammonium chloride to the mobile phase. Recoveries were near 100% from blood serum, 83–94% from muscle, and 80–100% from liver and kidney with sensitivities of 0.1 ppm or less for muscle and blood serum.

INTRODUCTION

Although a few methods have been described for determining tetracyclines in blood serum and tissue using high-performance liquid chromatography (HPLC)[1–9], the results have in practice been difficult to reproduce. Commercial bonded reversed-phase columns have been reported to vary greatly in their suitability for chromatography of the tetracyclines[10,11]. Knox and Jurand[12] noted that results were improved by capping residual silanols. Sharma and Bevill[2] described a lengthy column conditioning procedure. Tsuji *et al.*[13] found that satisfactory separations could be made on a polymeric hydrocarbon packing, but they were unable to obtain satisfactory separations on bonded ODS packings.

Extraction and cleanup of residues in animal tissues also present problems since tetracyclines do not readily partition into organic solvents from water. Solid-phase extraction using either XAD-2 resin[6,7] or bonded ODS cartridges[4,5] has been used for concentration and cleanup. Retention of tetracyclines on bonded ODS cartridges from different manufacturers was found to vary considerably[5]. Losses have been observed during concentration of samples by evaporation after elution from columns[7].

The present paper adresses the problems encountered by previous investigators. Tetracyclines were concentrated by solid-phase extraction directly on the ana-

lytical column which saves a great deal of time and eliminates the possibility of losses during evaporation. It was found that excellent resolution of tetracyclines could be obtained on an all-organic polymeric column. However, it was also found that by simply adding a silanol blocking agent (tetramethylammonium chloride) to the mobile phase, very similar results could be obtained on a bonded ODS column. These modifications have been incorporated into a simple rapid procedure which gives improved sensitivity, resolution and recovery of the tetracyclines.

MATERIALS AND METHODS

Chemicals and equipment

Equipment. Vortex mixer; blender; graduated cylinders, separatory funnels; 15-ml conical centrifuge tubes graduated to 0.1 ml. All glassware was cleaned in special detergents designed for critical cleaning and rinsed with 1% hydrochloric acid and distilled water before use.

Chemicals. Acetonitrile, methylene chloride, petroleum ether (b.p. 30–60°C) and methanol were residue analysis grade. Other chemicals were reagent grade. Chlortetracycline, tetracycline and oxytetracycline were from Sigma, tetramethylammonium chloride was from Eastman-Kodak and 1-heptanesulfonic acid (sodium salt) from Aldrich. Tetracycline stock solutions of 1, 0.1 and 0.01 $\mu g/\mu l$ were prepared weekly in 0.01 N hydrochloric acid and stored refrigerated.

HPLC apparatus. Varian Model 5000 liquid chromatograph, UV-50 detector, Valco automatic loop injector with 200-μl loop.

HPLC columns. Hamilton PRP-1, 10 μm, 250 mm × 4.1 mm I.D. used without a guard column; Varian Micropak SPC-18, 4 μm, 150 mm × 4.6 mm I.D., used with recommended guard column; Polymer Laboratories, PLRP-S 5 μm, 150 mm × 4.6 mm I.D., used with recommended guard column.

Procedures

Extractions. About 25 g of tissue were weighed accurately and blended with 3 volumes (v/w) of 1 N hydrochloric acid. Blood serum was diluted with 1 volume of 1 N hydrochloric acid and treated as for the tissue homogenate. Then 8 ml of homogenate or diluted blood serum were transferred to a 125-ml conical flask and 32 ml of acetonitrile were added with swirling. After standing for 5 min, the supernatant liquid was decanted through a plug of glass wool in the stem of a funnel and one-half volume (20 ml) of filtrate was collected, equivalent to 1 g of tissue. The filtrate was transferred to a separatory funnel, 20 ml of methylene chloride and 20 ml of petroleum ether were added and the mixture was shaken vigorously. The water layer was collected in a graduated tube. The volume was adjusted to 4 ml with water if necessary,.

Recovery experiments. Tissue —after weighing the tissue, an appropriate amount of tetracycline stock solution was added and allowed to equilibrate for 30 min before proceeding with the analysis. Blood serum —an appropriate amount of stock solution was added to the serum.

HPLC procedure. The aqueous layer was injected in 200-μl aliquots using the automatic loop injector until the desired volume was on the column. During the injection procedure, the solvent composition was 0.01 M phosphoric acid–methanol

(80:20) with a flow-rate of 1 ml/min. Then, 2 min after the final injection, a gradient of acetonitrile was started to a final proportion of 0.01 M phosphoric acid–acetonitrile–methanol (30:50:20), 25 min after the last injection (23 min after start of the gradient). Tetracyclines were monitored at 355 nm. The gradient was varied slightly with some samples, especially liver and kidney, to a final concentration of 30:40:30 (25 min) to improve separation from interferences.

RESULTS AND DISCUSSION

The use of all-organic reversed-phase columns was investigated for chromatography of tetracyclines because of reports of inconsistent results with bonded reversed-phase columns[10,11]. The polymeric columns are stable in the pH range 1–13, which is wider than for bonded columns[14]. Knox and Jurand[15] concluded that the best separations of tetracyclines were obtained in the pH range 1–2.5 but that bonded columns were frequently unstable in this pH range.

The use of citric acid (buffered to pH 2.2)–acetonitrile as recommended by the manufacturer for chromatography of tetracyclines on PRP-1 columns[16] was less satisfactory than 0.1 M citric acid as used by De Leenheer and Nelis[8] on bonded columns. Dilute phosphoric acid proved even more satisfactory and 0.01 M was better than 0.05 M. This gave baseline separation of the three tetracyclines and also resolved several minor unidentified components. Use of an acetonitrile gradient gave sharper peaks, especially for chloretracycline which was the last to elute, and markedly improved sensitivity for this compound. Methanol was relatively ineffective as an eluent compared with acetonitrile and gave much broader peaks. However, in working with tissue extracts, methanol was added to improve separations of tetracyclines from interferences. Tetracyclines were monitored by UV absorbance at 355 nm, a wavelength where absorbance in tissue extracts is slight. Sensitivity could be somewhat improved by monitoring at 280 nm but the advantage is offset by greater interference from other substances at this wavelength. Use of an ion-pairing reagent, sodium heptanesulfonate, was also evaluated. This increased the retention times but also decreased separations so that baseline separation of oxytetracycline and tetracycline was not achieved. Ion-pairing did not improve peak shape, sensitivity or separation from interferences.

When the same solvent systems (0.1 M citric acid or 0.01 M phosphoric acid) were used with a bonded reversed-phase column (Varian SPC-18) separations were poor and peaks were not sharp. It was subsequently found that results nearly equivalent to those obtained on a polymeric column could also be obtained on the bonded column by simply adding 0.01 M tetramethylammonium chloride to the 0.01 M phosphoric acid in the mobile base. Alkylammonium compounds have been used to improve chromatography of curare alkaloids[17] and tetracyclines[18]. The use of alkyl-substituted ammonium compounds as silanol blocking agents on bonded columns is discussed by Bij et al.[19]. With tetramethylammonium chloride in the mobile phase, the polymeric and bonded columns could be used virtually interchangeably. The bonded columns we have used shown no deterioration after prolonged use with 0.01 M phosphoric acid.

A number of procedures use fairly strong acid to dissociate tetracyclines from proteins in biological materials[3,7,9,20] using either hydrochloric[7,9,20] or trichloracetic

acid[3]. In the present procedure, blending tissue with 1 N hydrochloric acid was satisfactory. Use of weaker acid (0.1 N) did not give good recoveries. The homogenates were deproteinized with acetonitrile, a procedure used successfully with penicillins and tylosin[21]. After standing for 10 min, this gave a clear to slightly opalescent filtrate through a plug of glass wool.

Further concentration and cleanup of the filtrates were necessary for detection of tetracyclines at the 0.1 ppm tolerance level required in some tissues[22]. Tetracyclines did not readily partition into organic solvents. The best results with this approach were obtained from the water layer without pH adjustment using 1:1 ethyl acetate–acetonitrile which carried considerable water into the organic phase. Recoveries of chlortetracycline were 90–100% after evaporation of the organic phase under vacuum but recoveries of oxytetracycline were less than 70%. Extraction into ethyl acetate from a phosphate–sulfite buffer, as described by De Leenheer and Nelis[8] for doxycycline, was not effective with the other tetracyclines. Sharma et al.[1] and Sharma and Bevill[2] described methods for extraction of tetracyclines into ethyl acetate in the presence of calcium ion or calcium ion, barbital, and phenybutazone, respectively. The tetracyclines were re-extracted into acid, effecting some concentration which was, however, inadequate for determination in tissues at 0.1 ppm.

The tetracyclines could be concentrated in the water layer formed by adding equal volumes of petroleum ether and methylene chloride to the acetonitrile filtrate. Most pigments and lipids remained in the organic layer. With filtrates from liver and kidney, it was necessary to add additional acetonitrile to prevent emulsion formation. In this case recovery of tetracyclines was improved by extracting the organic layer with an additional 4 ml of water.

Solid-phase extraction on XAD-2 resin[6,7] or bonded cartridges[4,5] from an aqueous solution has been used. We compared the use of a short XAD-2 column with direct concentration on the analytical columns which are similar chemically to the XAD-2 resin or bonded cartridges used. Acetonitrile was much more effective in eluting tetracyclines from either the XAD-2 resin or the analytical columns than methanol as used by some other investigators[4,6,7]. More consistent results were obtained by concentration from successive injections on the analytical column. With up to 20% methanol in the mobile phase, tetracyclines did not move while much interfering material washed through the column. Any desired amount could be injected. This was also faster since it eliminated a separate step which required evaporation of the eluate from the XAD-2 column. The possibility of losses during evaporation of solvent was also eliminated.

An injection volume of 200 μl of the aqueous layer was used. Depending on the sensitivity sought, one or several injections could be made before the gradient was applied. Sensitivity was in practice limited by the sample background, which was negligible with muscle and blood serum but considerable with liver and kidney. Fig. 1 shows tetracycline standards, Fig. 2 shows a beef muscle blank (0.2 equiv.) and Fig. 3 shows beef muscle spiked with 1 ppm of oxytetracycline and chlortetracycline. Tetracycline, which was present as an impurity in the chlortetracycline standard, gave a distinct peak. Four 0.2-ml injections of sample extract were used for Figs. 2 and 3. Since official tolerances for various tetracyclines vary from 0.1 to 4 ppm depending on species and tissue[22] the amount of sample injected can be varied to give the required sensitivity. Since detection limits are *ca.* 10 ng for oxytetracycline or tetra-
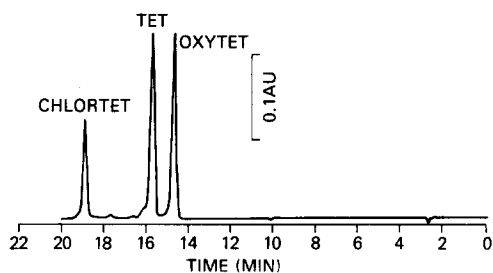
Fig. 1. Tetracycline standards (2 µg) on a Polymer Laboratories PLRP-S column. Varian Model 5000 liquid chromatograph, Valco automatic loop injector with 200-µl loop, Varian UV-50 detector set at 355 nm. Operating conditions: flow-rate, 1 ml/min; program, 0.01 M phosphoric acid–acetonitrile–methanol, 80:0:20 (0–2 min) to 30:50:20 (25 min).

cycline and 20 ng for chlortetracycline, the equivalent of 0.2 g (ml) of sample must be injected for detection of 0.1 ppm. Table I summarizes recoveries from spiked pork and Table II from beef tissue and blood serum. Recoveries from blood serum are essentially quantitative and the observed variations probably result from a slight drift in peak response sometimes observed. It is possible that peak area might be superior to peak height for quantitation. Recoveries are somewhat lower and slightly less consistent with tissue samples. Again, some of the variability probably results from drift of peak response which may be affected by other materials in sample extracts. However, recoveries particularly of chlortetracycline, are markedly higher than were reported by Onji et al.[7] and precision is comparable. Recoveries are also somewhat higher than were reported by Oka et al.[5]. Both recoveries and precision are well within acceptable limits for residue analysis[23].

Concentration on the column saves time and improves recoveries. No time-consuming evaporative steps are required. The analysis time for a single sample is ca. 60–90 min.
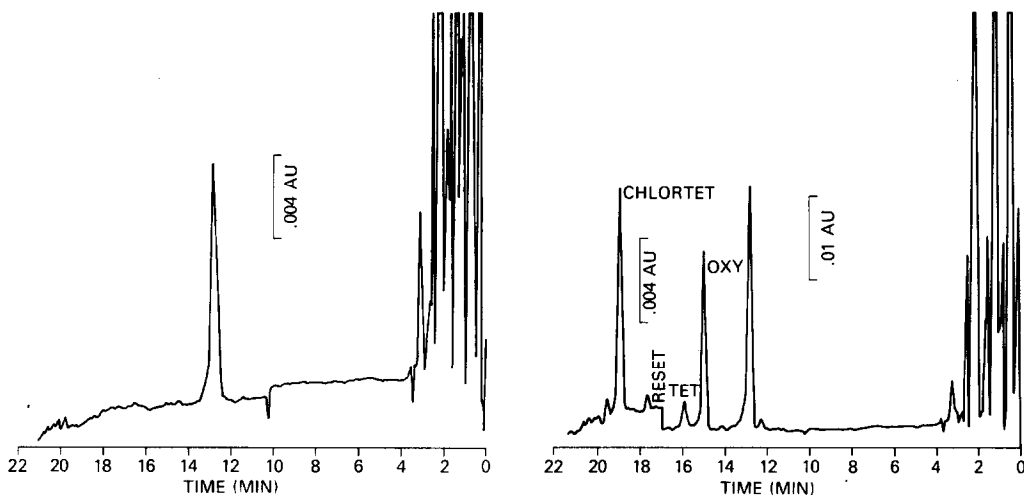

Fig. 2. Beef muscle bank; operating conditions as in Fig. 1. Four 200-µl aliquots of sample extract injected, equivalent to 0.2 g muscle.

Fig. 3. Same as Fig. 2 but spiked with 1 ppm chlortetracycline and oxytetracycline.

TABLE I

RECOVERY OF TETRACYCLINE ADDED TO PORCINE BLOOD SERUM AND TISSUES

Sample	Added (ppm)	Tetracycline recovery (%)	Oxytetracycline recovery (%)	Chlortetracycline recovery (%)
Muscle	1	96	89	92
	1	82	102	100
	1	97	103	95
	1	94	100	93
	10	91	95	98
	10	96	95	93
Mean ± S.D.		92.7 ± 5.2	98.5 ± 4.5	95.2 ± 2.9
Blood serum	10	95	101	102
	10	107	—	—
	1	97	99	97
	1	99	97	97
Mean ± S.D.		99.5 ± 4.6	99.0 ± 1.6	98.7 ± 2.4
Liver	10	78	103	77
	10	71	94	85
Kidney	10	79	88	93
	10	78	86	103

TABLE II

RECOVERY OF TETRACYCLINE ADDED TO BOVINE TISSUES AND BLOOD SERUM

Tissue	Added (ppm)	Tetracycline recovery (%)	Oxytetracycline recovery (%)	Chlortetracycline recovery (%)
Muscle	1	84	82	99
	1	77	101	82
	1	86	83	84
	1	90	95	91
	10	90	97	87
	10	79	101	95
Mean ± S.D.		84.3 ± 5.0	93.2 ± 7.8	89.7 ± 6.0
Blood serum	1	105	106	91
	1	103	95	95
	1	100	102	91
	10	102	97	100
	10	102	98	100
	10	100	98	100
Mean ± S.D.		102.0 ± 1.7	99.3 ± 3.6	96.2 ± 4.1
Liver	10	88	90	88
	10	79	86	82
Kidney	10	81	106	95
	10	—	104	95

For blood serum, this method requires more time than that of Nilsson-Ehle *et al.*[3] but is capable of substantially greater sensitivity since more sample can be injected.

Use of an all-organic polymeric column or addition of a silanol blocking agent to the mobile phase give reproducible chromatographic results, thus removing a major stumbling block in analysis of tetracyclines.

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