Journal of Chromatography, 166 (1978) 213–220

CHROM. 11,238

IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURE FOR THE DETERMINATION OF TETRACYCLINES IN PLASMA, URINE AND TISSUES

J. P. SHARMA and R. F. BEVILL*

University of Illinois at Champaign-Urbana, College of Veterinary Medicine, Division of Pharmacology and Toxicology, Urbana, Ill. 61801 (U.S.A.)

(Received April 18th, 1978)

SUMMARY

An improved extraction procedure for the determination of oxytetracycline, tetracycline and chlortetracycline in urine, plasma and tissues by high-performance liquid chromatography is described. The addition of phenylbutazone (3,5-dioxo-1,2-diphenyl-4-*n*-butylpyrazolidine) to water, urine or plasma enhances the extraction of these compounds by ethyl acetate. The recovery of oxytetracycline from plasma is increased six fold and the need for two separate extractions of urine and plasma is eliminated. The formation of phenylbutazone–tetracycline ion pairs and their role in the extraction process is discussed.

INTRODUCTION

An assay procedure capable of detecting part-per-million (ppm) concentrations of oxytetracycline, chlortetracycline, and tetracycline in the plasma and urine of domestic food-producing animals was recently reported[1]. However, when swine, sheep and cattle were dosed with recommended doses (5 mg/lb. body weight) of these drugs, we were unable to detect the drug in plasma after the twelfth post-dosing hour. In order to further define the pharmacokinetics of tetracyclines in food-producing animals and to study the relationship between the plasma and tissue concentrations of these drugs, an assay having increased sensitivity was required.

There was considerable evidence[2–6] that a portion of the tetracyclines present in the plasma of animals treated with these drugs were bound to plasma proteins and as such were unavailable for extraction into organic solvents. Since phenylbutazone (3,5-dioxo-1,2-diphenyl-4-*n*-butylpyrazolidine) has been reported to competitively displace certain drugs from sites of protein binding[7], we decided to incorporate phenylbutazone in the extraction step of the assay procedure and study its effect on the extraction of tetracyclines from plasma.

The purpose of this paper is to (1) report an improved extraction procedure

* To whom correspondence should be addressed.

for tetracyclines in urine and plasma, (2) describe a method for the extraction and quantitation of tetracyclines in tissue, and (3) suggest the role played by phenylbutazone during the extraction.

EXPERIMENTAL

Apparatus

The high-performance liquid chromatographic (HPLC) system and chromatographic conditions described previously[1] were used in this study.

Conditioning of columns

New columns (μBondapak C_{18}; Waters Assoc., Milford, Mass., U.S.A.) must be properly conditioned to reproduce the results reported in this and an earlier paper[1]. Recorded peaks corresponding to the elution of tetracyclines evidenced marked tailing following the injection of these compounds on new, unconditioned columns. To correct this problem, it was necessary to flush the new column with methanol (4–5 h), an aqueous solution of 0.05 *M* disodium ethylenediamine–tetraacetic acid adjusted to pH 7.0 (10–12 h), water–acetonitrile (50:50, v/v) containing 10 mg chlortetracycline per 100 ml (2–3 h), acetonitrile–water (50:50, v/v) (2–3 h) and the mobile phase (2–3 h). During the conditioning process the solvent operating pressure increased 300–400 p.s.i. Since the selectivity of the column packing may vary from column to column, it may be necessary to optimize tetracycline elution from individual columns. This can be accomplished by making slight adjustments in the concentration of acetonitrile or in the pH (2.1–2.4) of the mobile phase.

Reagents and materials

The mobile phase, extraction reagents and tetracycline standards used in this study were described in a previous report[1].

An aqueous solution of phenylbutazone (200 mg/ml) was used as received (Norden Labs., Lincoln, Nebr., U.S.A.). Although the solution contained small amounts of benzyl alcohol and sodium hydroxide, these compounds did not interfere with the extraction nor subsequent chromatographic procedures.

Extraction procedures and preparation of standard curves for plasma and urine

A 1-ml portion of tetracycline working standard was added to glass tubes containing 2.0 ml of plasma or 3.0 ml of urine. The resulting fortified solutions contained 0.1–5 μg tetracycline per ml. Sodium barbital (0.08 *M*), calcium chloride solution (4%, w/v), phenylbutazone, and ethyl acetate (1, 1, 0.15 and 8 ml, respectively) were added to the tubes containing fortified urine or plasma. The tubes were agitated for 5 min and then centrifuged for 10 min at 1000 *g*. Portions of 7 ml of the ethyl acetate were subsequently transferred to tubes containing 0.5 ml of phosphoric acid (1.0 *N*). These tubes were agitated for 3 min and then centrifuged for 3 min at 1000 *g*. The ethyl acetate layer was discarded and 8 μl of the acid layer were injected on the octadecylsilane column. Each analysis was performed in duplicate and standard curves were prepared by plotting the concentrations of individual tetracyclines (μg/ml) *versus* average recorded peak heights.

Tissue. Portions of liver, kidney or skeletal muscle were cut into 2-cm cubes.

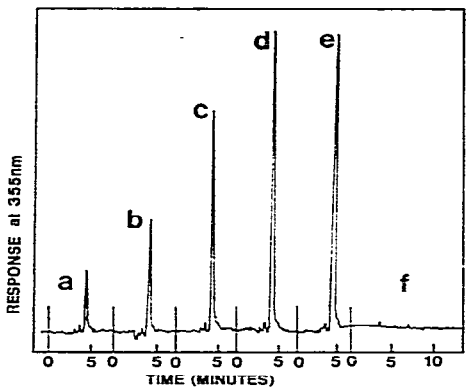
The cubed tissue (100 g) was placed in stainless-steel blender cups and injected with known volumes of solutions containing standard amounts of a tetracycline. The cubed, injected tissue was immediately frozen in liquid nitrogen, blended to a fine powder (Blender 7012S, Waring, New Hartford, Conn., U.S.A.) and stored at −20°. Several lots of tissue containing 0.2–5 μg of chlortetracycline, tetracycline or oxytetracycline were prepared in this manner.

Samples of 10 g of the tissue powder were rapidly weighed into plastic weighing boats and transferred to a 125-ml glass stoppered erlenmeyer flask. Thirty milliliters of ethyl acetate (tetracycline or oxytetracycline extraction) or dichloromethane (chlortetracycline extraction), sodium barbital (1.0 ml), calcium chloride (1.0 ml) and phenylbutazone (0.5 ml) were added to each of the flasks which were then agitated on a mechanical shaker for 15 min. The flask contents were filtered on glass wool supported in a glass funnel and the filtrate was collected in a 100-ml distillation flask. The erlenmeyer flasks were rinsed twice with 10 ml of the solvent and the washings were passed through the glass wool and collected in the distillation flasks. The solvent was removed under reduced pressure at a temperature of 46–48° (30–32° when dichloromethane was used). Distillation was interrupted when approximately 0.5 ml of viscous liquid remained in the flask. The flask contents were dissolved in ethyl acetate (5.0 ml) and transferred to screw-capped glass tubes. The distillation flasks were rinsed with a second volume of ethyl acetate (3.0 ml) which was added to the tubes. Water (1.0 ml) was added to each tube to remove excess phenylbutazone from the extraction solvent and prevent its precipitation during the reextraction process. The tubes were agitated for 5 min and then centrifuged for 5 min at 1000 *g*. The remainder of the extraction and quantitation procedure was performed as previously described for urine and plasma.

RESULTS AND DISCUSSION

Dramatic increases in the extraction of tetracyclines from plasma and urine occurred when the extraction was carried out in the presence of phenylbutazone. To understand the nature of the specie being extracted into the organic phase, the influence of calcium chloride, sodium barbital and phenylbutazone, singly or in combination, on the extraction of oxytetracycline from urine was studied (Fig. 1). No oxytetracycline was detected in the ethyl acetate extracts of oxytetracycline fortified urine to which calcium chloride had been added. The addition of sodium barbital and calcium chloride to oxytetracycline fortified urine increased the efficiency of the extraction process but even so the extraction was less efficient than that obtained when only phenylbutazone was added to the urine prior to extraction. Extraction was most efficient when calcium chloride and phenylbutazone were added to urine prior to the extraction process. Similar results were obtained when plasma was used.

When the above experiments were repeated using aqueous solutions of oxytetracycline, the relative efficiency of extraction was about the same as that obtained using plasma or urine (Fig. 2b, c). Since oxytetracycline was extracted equally well from water and plasma in the presence of phenylbutazone, we concluded that phenylbutazone increased extraction efficiency in some manner other than by competitive displacement of bound drug from sites of protein binding.



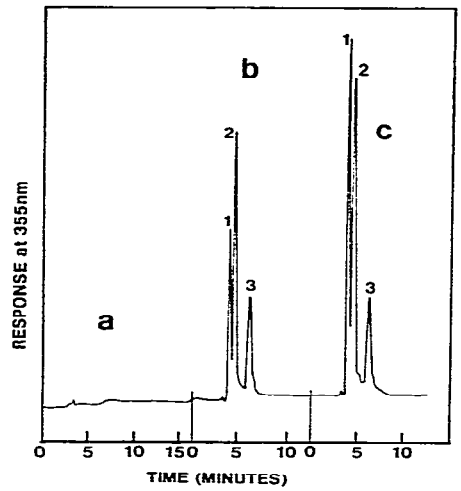
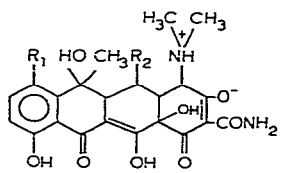


Fig. 1. Chromatograms obtained using mobile phase A[1] when swine urine containing 10 μg/ml oxytetracycline was extracted in the presence of (a) calcium chloride and barbital, (b) phenylbutazone and barbital, (c) phenylbutazone, (d) calcium chloride and phenylbutazone, (e) calcium chloride, phenylbutazone and barbital. A chromatogram of control urine extracted in the presence of calcium chloride, phenylbutazone and barbital is presented (f).

Fig. 2. Chromatograms obtained using mobile phase A after (a) control plasma containing calcium chloride, phenylbutazone and barbital; (b) water containing calcium chloride, barbital and (1) 9.9 μg/ml oxytetracycline, (2) 7.7 μg/ml tetracycline, (3) 11.5 μg/ml chlortetracycline; (c) water containing calcium chloride, barbital and phenylbutazone and (1) (2) and (3) were extracted.

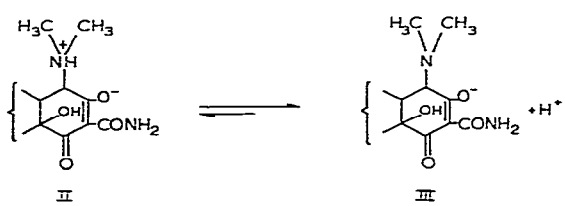
The acid salts of tetracyclines have three dissociation constants (Table I)[8–10]. Stephens *et al.*[10] determined that tetracycline bases exist as dipolar ions (I) when present in aqueous solutions at pH 4.0–7.0.



I

	R_1	R_2
Oxytetracycline	H	OH
Tetracycline	H	H
Chlortetracycline	Cl	H

However, as the pH of the solution containing tetracyclines is increased to 8–9, marked dissociation of the dimethylammonium cation occurs (II, III).



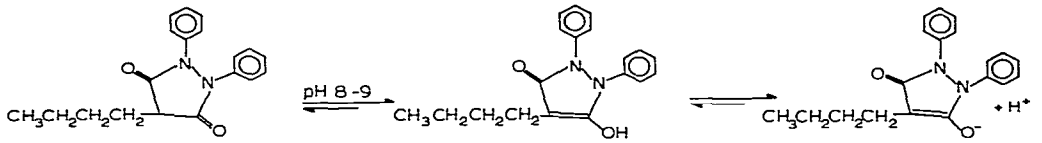
II ⇌ III (1)

When phenylbutazone (IV) is added to the tetracycline solution (pH 8.0–9.0) it exists primarily as an ionized enol (VI) which subsequently forms an ion pair with the undissociated dimethylammonium cation (II).

TABLE I

pK_a VALUES OF ACID SALTS OF TETRACYCLINES[10]

	pK_a		
	C_3–OH	$-\overset{\overset{+}{\vert}}{N}-H$	C_{12}–OH
Oxytetracycline	3.27	7.32	9.11
Tetracycline	3.30	7.68	7.68
Chlortetracycline	3.30	7.44	9.27



IV V VI (2)

Thus it would appear that limited ion-pair formation occurs under these conditions. However, when an organic phase is added to the mixture, the ion pairs are extracted from the aqueous phase. The removal of ion pairs from the aqeous phase shifts the equilibrium to the left in eqn. 1 and to the right in eqn. 3 and ion-pair formation continues until the extraction is complete.

$$\text{II} + \text{VI} \rightleftharpoons \underset{\text{ion pair}}{(\text{II}\cdot\text{VI})} \tag{3}$$

The C_{11} and C_{12} functional groups of tetracyclines are sites of complex formation with calcium[10–12]. At pH 8.0–9.0 the enolic hydrogen at C_{12} undergoes sufficient ionization to allow the formation of a calcium chelate. Thus, it would appear that calcium chelation and phenylbutazone–tetracycline ion-pair formation occur simultaneously under the experimental conditions described earlier and that the actual specie being extracted into the organic phase is the ion pair formed between calcium complexes of tetracyclines and phenylbutazone. The primary use of sodium barbital was to maintain the pH of the extraction at 8–9. However, since barbital facilitated the extraction of the calcium complexes of tetracyclines, we concluded that barbital also formed an ion pair with these compounds. Under the conditions described barbital and phenylbutazone anions competed for available tetracyclines, but at equilibrium the formation of the tetracycline–phenylbutazone ion pair predominated. The magnitude of the effect of phenylbutazone in the extraction of the three tetracyclines was dependent on the difference in the hydrophobicity of the three tetracyclines themselves. The ability of tetracyclines to form ion pairs with anions has been described previously[13].

The suitability of various acids (hydrochloric, sulfuric, nitric, phosphoric, perchloric and acetic) for the reextraction of tetracyclines from ethyl acetate was

TABLE II

DETERMINATION OF TETRACYCLINES IN URINE, PLASMA AND TISSUE

All the recovered values are the average of two separate determinations.

Tetracyclines	*Swine urine*			*Swine plasma*			*Swine liver*		
	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/g)*	*R.S.D. (%)*
Oxytetracycline	0.27	0.26	0.0	0.40	0.45	0.0	1.0	0.95	7.0
	0.55	0.54	0.0	1.0	1.01	1.0	4.0	3.95	2.0
	2.21	2.17	1.6	3.0	2.96	3.0	5.0	5.00	1.4
Tetracycline	0.25	0.24	0.0	0.48	0.46	0.0	0.54	0.55	10.0
	0.52	0.50	0.0	0.96	0.96	5.2	1.07	0.97	3.5
	1.00	1.00	4.0	1.92	1.90	0.0	5.30	5.17	2.5
Chlortetracycline	0.85	0.83	1.0	0.96	0.97	1.5	0.50	0.65	10.5
	1.69	1.63	0.0	1.92	1.97	2.0	1.05	1.12	3.5
	3.40	3.44	0.4	3.84	3.88	3.0	5.0	5.27	3.5

examined. Sulfuric and phosphoric acids proved most effective whereas acetic acid was least effective. Knox and Jurand[13] reported that certain anions formed ion pairs with and affected the chromatographic behavior of tetracyclines. Their observations may explain the failure of perchloric acid to reextract tetracyclines from ethyl acetate inasmuch as perchlorate–tetracycline ion pairs were highly soluble in the organic phase.

Linear standard curves (μg/ml *versus* peak height) were obtained when the modified method was used to determine the concentration of oxytetracycline, tetracycline, and chlortetracycline in plasma and urine fortified with these compounds (Fig. 3). The limits of assay sensitivity for oxytetracycline, tetracycline, and chlortetracycline in urine were 0.25, 0.25 and 0.5 μg/ml, respectively, whereas for plasma they were 0.5, 0.5 and 0.7 μg/ml respectively. The results obtained from the determinations of tetracyclines in swine urine and plasma are presented in Table II. Although a standard injection volume (8 μl) was used in this study, increased assay sensitivities can be obtained by increasing the injection volume to 20 μl.

The absolute recovery of tetracyclines from tissue was less than that obtained from urine and plasma and larger amounts of tissue (10 g) were extracted in order to obtain the desired level of assay sensitivity (0.5 μg/g). When ethyl acetate was used to extract chlortetracycline from tissues the drug epimerized during the distillation step (48°). However, no epimerization was noted during the distillation step (32°) when dichloromethane was used for extraction. Since dichloromethane proved as effective as ethyl acetate in extracting tetracyclines from tissue, its use is recommended for the extraction of chlortetracycline from tissues. The standard curves obtained when the concentration of oxytetracycline, chlortetracycline and tetracycline

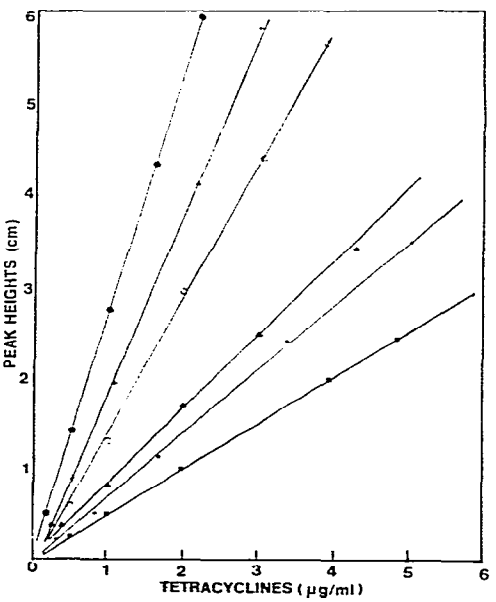
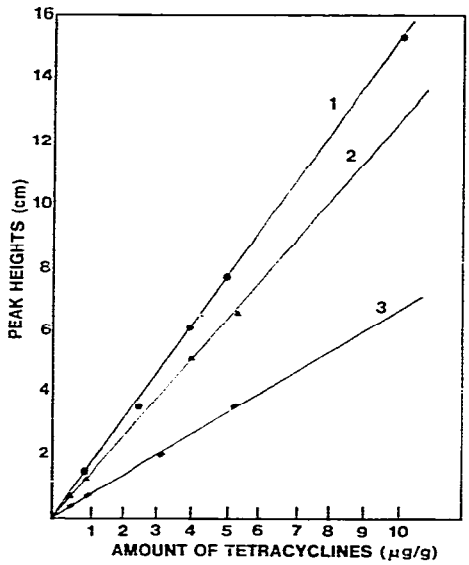


Fig. 3. Standard curves for the determination of tetracyclines in swine urine and plasma. ●, Tetracycline in urine; ⊗, oxytetracycline in urine; ○, tetracycline in plasma; ▲, oxytetracycline in plasma; •, chlortetracycline in urine; ■, chlortetracycline in plasma.



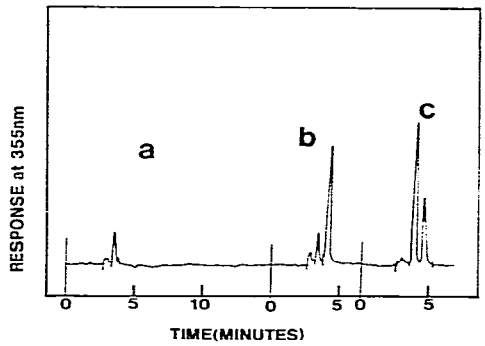


Fig. 4. Standard curves for the determination of tetracyclines in tissue (swine liver). ●, Oxytetracycline; ▲, tetracycline; ■, chlortetracycline.

Fig. 5. Chromatograms of control and drug fortified swine liver extracts. (a) Control; (b) oxytetracycline with mobile phase A; (c) tetracycline and chlortetracycline with mobile phase B. Recorded using a Hewlett-Packard 3380A intergrater; chart speed 30 cm/h; slope sensitivity 0.3 mV/min; attenuation 1.

in pork liver fortified with these drugs were determined are presented in Fig. 4. The recoveries of the drugs from fortified liver samples are listed in Table II. Chromatograms of control and fortified tissue extracts are shown in Fig. 5.

The main advantages of this method over the method we have previously reported[1] are: (1) a six-fold increase in the sensitivity of the assay for oxytetracycline, (2) a single, uniform extraction procedure replaces the two separate procedures previously required for the extraction of urine and plasma and (3) the need for two extractions of oxytetracycline from urine and plasma has been eliminated.

REFERENCES

1 J. P. Sharma, E. G. Perkins and R. F. Bevill, *J. Chromatogr.*, 134 (1977) 441.
2 C. M. Kunin, A. C. Dornbush and M. Finland, *J. Clin. Invest.*, 38 (1959) 1950.
3 S. M. V. Witteman and R. Yeary, *J. Pharmac. Exp. Ther.*, 140 (1963) 258.
4 C. W. Kunin, *Proc. Soc. Exp. Biol. Med.*, 110 (1962) 311.
5 J. E. Rosenblatt, J. E. Barrett, J. L. Brodie and W. M. N. Kirby, *Antimicrob. Ag. Chemother.*, 6 (1966) 134.
6 N. H. Steigbiegel, C. W. Reed and M. Findland, *Amer. J. Med. Sci.*, 255 (1968) 296.
7 D. M. Woodbury and E. Fing, in L. S. Goodman and A. Gilman (Editors), *The Pharmacological Basis of Therapeutics*, MacMillan, New York, 5th ed., 1975, p. 340.
8 F. A. Hochstein, C. R. Stephens, I. H. Contover, P. P. Regna, R. Pasternack, P. N. Gordon, F. J. Pilgrim, K. J. Burnings and R. B. Woodward, *J. Amer. Chem. Soc.*, 75 (1953) 5455.
9 A. Albert, *Nature (London)*, 172 (1953) 201.
10 C. R. Stephens, K. Mural, K. J. Brunings and R. B. Woodward, *J. Amer. Chem. Soc.*, 78 (1956) 4155.
11 J. T. Dolusio, *J. Med. Chem.*, 6 (1963) 16.
12 E. H. Gans and T. Higuchi, *J. Amer. Pharm. Ass.*, 8 (1957) 458.
13 J. H. Knox and J. Jurand, *J. Chromatogr.*, 110 (1975) 103.