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Separation and Quantitation of Chlortetracycline, 4-Epitetracycline, 4-Epianhydrotetracycline, and Anhydrotetracycline in Tetracycline by High-Performance Liquid Chromatography

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Received September 7, 1979, from *Bristol Laboratories, Division of Bristol-Myers Co., Syracuse, NY 13201.* Accepted for publication March 12, 1980.

Abstract □ The analysis of tetracycline epimers in tetracycline preparations by high-performance liquid chromatography is described. The method uses a microparticulate phenyl column (3.9 mm i.d. × 30 cm) with a step gradient of 12–22% acetonitrile in 0.2 *M* phosphate buffer at pH 2.2. The analysis takes 22 min. The relative standard deviations of the method (2σ, $n = 6$) for the analysis of chlortetracycline, 4-epitetracycline, 4-epianhydrotetracycline, and anhydrotetracycline in tetracycline were ±3.68, ±4.47, ±7.60, and ±2.77%, respectively.

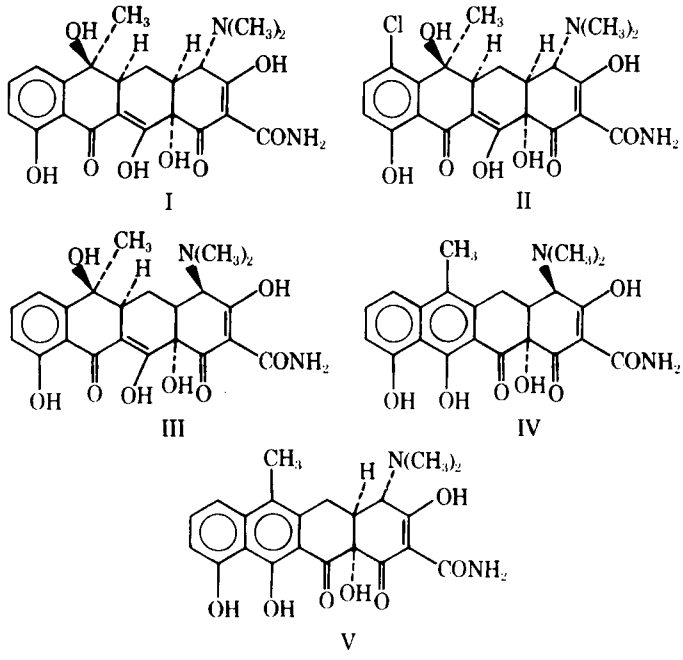
Keyphrases □ Tetracycline—simultaneous high-performance liquid chromatographic analysis of tetracycline epimers in tetracycline preparations □ High-performance liquid chromatography—analysis, tetracycline epimers in tetracycline preparations □ Antibacterials—tetracycline, high-performance liquid chromatographic analysis of tetracycline epimers in tetracycline preparations

Pharmaceutical preparations of tetracycline (I) contain small quantities of related compounds as impurities. The most important compounds are chlortetracycline (II), 4-epitetracycline (quatrimycin, III), 4-epianhydrotetracycline (IV), and anhydrotetracycline (V). Their permitted concentrations are listed in the Federal Register (1) for tetracycline dosage forms marketed in the United States and in the British Pharmacopoeia (2) and European Pharmacopoeia (3) for the European markets. The need for a simple assay prompted this investigation of the conditions under which these compounds could be separated and quantitated by high-performance liquid chromatography (HPLC) without a solvent gradient system.

BACKGROUND

TLC (4, 5), paper chromatography (6), and column chromatography followed by UV spectrophotometry (7, 8) have proved laborious and generally are not sufficiently sensitive or precise. A GLC method (9) requires prior formation of the trimethylsilyl derivative under carefully controlled conditions.

Several HPLC methods have been applied to separate III–V from I using a cation-exchange column with an ethylenediaminetetraacetic acid buffer (10), a C_{18} column (5–7% loading) with phosphate buffer and a linear gradient of 10–60% acetonitrile (11), a C_{18} column (10–18% loading) with a linear gradient of methanol–water–0.2 *M* phosphate buffer at pH 2.5 (30:60:10) and methanol–acetonitrile–water–0.2 *M* phosphate buffer at pH 2.5 (50:30:20:10) (12), and a C_{18} column with water–acetonitrile–perchloric acid in two steps (13).



In the present study, a microparticulate phenyl column with a step gradient of 12–22% acetonitrile in 0.2 *M* phosphate buffer at pH 2.2 (using an automatic switching valve) at a flow rate of 2.6 ml/min was used to separate II–V from I in 22 min. The method did not require a solvent gradient system and needed only one pump. It was tested for the analysis of several different tetracycline formulations.

EXPERIMENTAL

Apparatus—The liquid chromatographic apparatus shown in Fig. 1 was used[1].

Reagents and Materials—Ammonium 4-epitetracycline[2], 4-epianhydrotetracycline hydrochloride[2], anhydrotetracycline hydrochloride[2], chlortetracycline hydrochloride[3], acetonitrile[4], phosphoric acid[5], potassium hydroxide[5], and ammonium hydroxide[5] were used.

[1] A Valcor series SV-72 automatic switching valve, a Waters Associates model 6000 A pump, a Valco loop injector, a Waters Associates μBondapak phenyl column, and a Waters Associates model 440 absorbance detector were used.
[2] Bristol reference standards, Bristol Laboratories, Syracuse, N.Y.
[3] USP reference standard.
[4] Burdick & Jackson Laboratories, Muskegon, Mich.
[5] Fisher Scientific Co., Fair Lawn, N.J.

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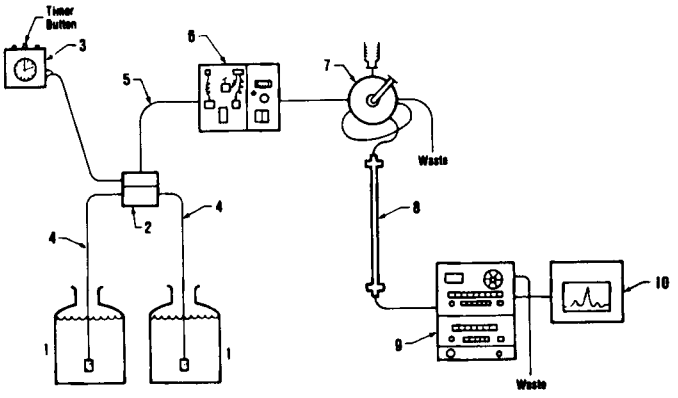

Figure 1—*HPLC step-gradient system for determination of tetracycline epimers. Key: 1, 2-liter reservoirs containing Eluents 1 and 2; 2, automatic switching valve (miniature three-way solenoid); 3, delay timer; 4, polytef tubing (3.2 mm i.d.) used to transfer the eluent from the reservoirs to the switching valve; 5, polytef tubing (16 cm long × 3.2 mm i.d.) used to transfer the eluent from the valve to the pump; 6, pump; 7, 20-μl loop injector operated at 7000 psi; 8, μBondapak phenyl 3.9 mm i.d. × 30-cm column; 9, absorbance detector; and 10, recorder.*

Mobile Phase—Eluent 1 was prepared by adding 240 ml of degassed acetonitrile and 27 ml of phosphoric acid to 1650 ml of degassed, distilled, deionized water. The pH was adjusted to 2.2 with 45% aqueous KOH, and the solution was diluted to 2 liters. The solution was degassed for only 15 sec prior to use.

Eluent 2 was prepared by adding 440 ml of degassed acetonitrile and 27 ml of phosphoric acid to 1500 ml of degassed, distilled, deionized water. The pH was adjusted to 2.2 with 25% aqueous KOH, and the solution was diluted to 2 liters with degassed, distilled, deionized water. The solution was degassed for only 15 sec prior to use.

Standard Solution Preparation—About 20 mg of ammonium 4-epitetracycline, 3 mg of epianhydrotetracycline hydrochloride, 3 mg of anhydrotetracycline hydrochloride, and 10 mg of chlortetracycline hydrochloride reference standards were weighed accurately into a 50-ml volumetric flask, dissolved, and diluted to volume with distilled water. Sonication sometimes was necessary for dissolution.

Sample Preparation—Bulk tetracycline hydrochloride samples were prepared by weighing accurately 500 mg of the sample into a 50-ml volumetric flask. The sample was dissolved and diluted to volume with distilled water.

Tetracycline base samples were prepared by weighing accurately 500 mg of the sample into a 50-ml volumetric flask. The sample was dissolved and diluted to volume with 0.1 *N* aqueous HCl.

Samples of hard, filled capsules containing sodium polymetaphosphate, phenazopyridine hydrochloride, sulfamethizole, and tetracycline phosphate were prepared by weighing accurately 500 mg of capsule blend into a 50-ml volumetric flask. To the flask were added 15 ml of distilled water and 1 ml of concentrated ammonium hydroxide. The flask was shaken until dissolution occurred. The solution was diluted to volume with pH 4.0 phosphate buffer, allowed to stand for 10 min, and filtered.

Sample Analysis—The column was conditioned with Eluent 1. The delay timer (Fig. 1) was set at 10 min. Twenty microliters of the standard solution or sample solution was injected using a loop. The timer was started, and the system was allowed to run. After 10 min, the automatic switching value opened to Eluent 2. The system was allowed to run on Eluent 2 until all peaks eluted (~15 min).

Calculations—The areas of the peaks were measured by the peak height times the half-width method or by a computer. The percentage of each compound was obtained from:

$$\text{factor for II, IV, and V: } F = \frac{\text{peak area of standard}}{\text{mg of standard}} \quad \text{(Eq. 1}a\text{)}$$

or:

$$\text{factor for III: } F = \frac{\text{peak area of standard}}{\text{mg of standard} \times 1.04} \quad \text{(Eq. 1}b\text{)}$$

and the correction factor for tetracycline based on the label claim (K):

$$K = \frac{\text{mg of sample}}{\text{label claim of tetracycline (mg)}} \quad \text{(Eq. 2)}$$

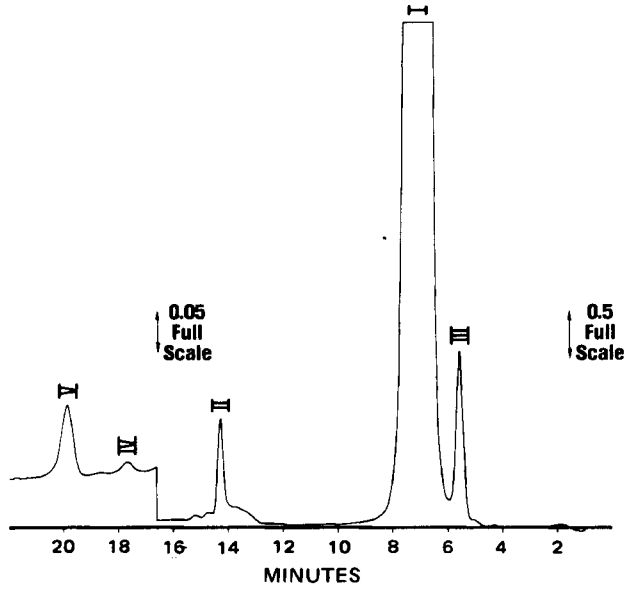

Figure 2—*Representative chromatogram of sample.*

so that:

$$\%\ \text{impurity} = \frac{\text{peak area of individual impurity} \times 100 \times K}{\text{mg of sample} \times F} \quad \text{(Eq. 3)}$$

DISCUSSION

The described HPLC method was developed for use in laboratories without a programmed solvent gradient system. The method requires one pump and an automatic switching valve. The microparticulate phenyl column produced symmetrical peaks without any tailing as compared to the C_{18} (alkyl) column (Figs. 2 and 3).

Standard linearity was checked over the ranges of 0.114–0.306 mg/ml for II, 0.286–0.620 mg/ml for III, 0.0215–0.036 mg/ml for IV, and 0.021–0.084 mg/ml for V. All four compounds responded linearly at the reported concentrations, thus providing more than an adequate range for their analysis as impurities in tetracycline (I).

Chromatographic variability was determined by six identical injections of a solution containing reference standards II–V. Standard variability was determined by a single injection of six different solutions containing reference standards II–V. Sample variability was determined by a single injection of six different preparations of the sample. Procedural variability was determined by a single injection of six individual standards and sample preparations. Standard deviations (s%) and relative standard deviations ($2s$%) for chromatographic variability, standard variability, sample variability, and procedural variability for II–V are shown in Table I.

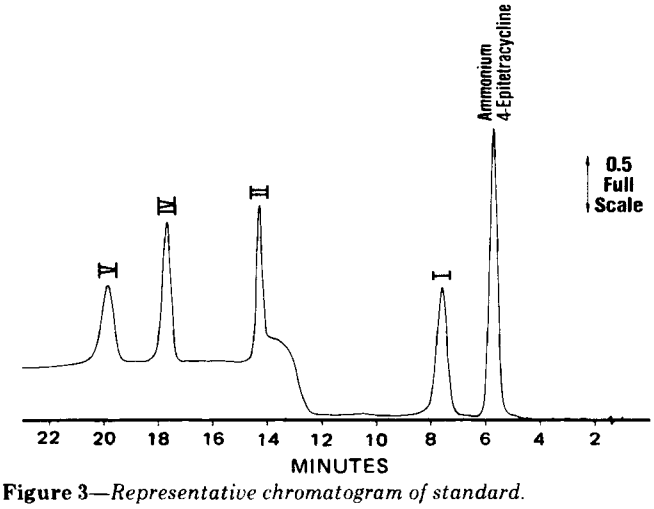

Figure 3—*Representative chromatogram of standard.*

Table I—Standard Deviations and Relative Standard Deviations

	II		III		IV		V	
	s%	$2s$%	s%	$2s$%	s%	$2s$%	s%	$2s$%
Chromatographic variability	0.0075	5.83	0.0663	2.80	0.0083	1.27	0.0183	3.39
Standard variability	0.0040	1.79	0.0081	2.34	0.0051	6.58	0.0016	2.20
Sample variability	0.029	5.44	0.049	4.92	0.0002	8.11	0.0012	4.10
Procedural variability	0.023	3.68	0.044	4.47	0.00017	7.60	0.0008	2.77

Table II—Representative Samples [a]

Sample[b]	II, %	III, %	IV, %	V, %
Capsule 1	1.95	1.13	1.30	3.19
Capsule 2	1.08	1.08	0.05	0.16
Capsule 3	1.98	1.08	0.83	4.01
Capsule 4	2.07	1.05	0.06	0.22
Capsule 5	2.05	1.12	0.02	0.13
Powder 1	1.88	1.09	0.004	0.068
Powder 2	1.99	1.05	0.005	0.054

[a] Percentages are based on the labeled claim of tetracycline. [b] Hard, filled capsules and bulk powders were used.

Accuracy of the assay was determined by spike recoveries of impurities II–V from I. Tetracycline (I) was spiked with impurities at 100% of the target value. The percent recoveries were 96, 102, 104, and 85% for II, III, IV, and V, respectively.

Several tetracycline-containing products were analyzed for impurities (II–V) (Table II).

In summary, the reversed-phase HPLC method described in this paper provides a rapid, sensitive, simple, and quantitative method for the simultaneous determination of impurities (II–V) in tetracycline (I).

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Aminoalkanesulfonic Acids IV: Synthesis of Mitodepressive *N*-Nitrosoaminoalkanesulfonic Acids

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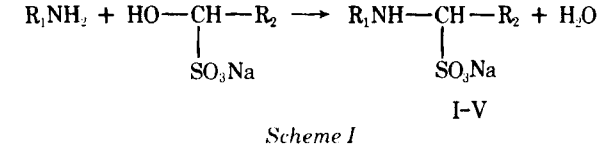
Received September 5, 1978, from the *Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovačića 1, Zagreb, Yugoslavia.* Accepted for publication March 12, 1980.

Abstract □ Sodium aminoalkanesulfonates were synthesized from substituted amines and aldehyde sodium bisulfites at 40–60°. Nitrosation yielded the corresponding *N*-nitroso derivatives. Mitodepressive activity on cresse (*Lepidium sativum* L.) was determined, and all compounds examined possessed growth inhibition activity. The *N*-nitroso derivatives were slightly less active but also showed less toxicity than the parent aminoalkanesulfonic acids.

Keyphrases □ Aminoalkanesulfonic acids—synthesis of *N*-nitroso derivatives, evaluation for mitodepressive activity □ Mitodepressive activity—evaluation of aminoalkanesulfonic acids and *N*-nitroso derivatives □ *N*-Nitrosoaminoalkanesulfonic acids—synthesis, evaluation for mitodepressive activity

Aminoalkanesulfonic acids are known to have antiviral (1, 2) and anticancer (3, 4) activities. Some *N*-nitroso compounds, such as *N*-nitrosomethylamine, *N*-nitrosodimethylamine, and *N*-nitrosodiethylamine, showed blastogenic action when applied on Hungarian hamsters (5). Some experiments (6) showed imbalances in DNA and histone synthesis during carcinogenesis induced by nitrosamines. On the other hand, some *N*-nitroso compounds, such as 1,3-bis(2-chloroethyl)-1-nitrosourea (carmustine), are useful in the treatment of certain malignant diseases (7).



Scheme I

These findings prompted the synthesis of some new derivatives in this series (8–10) to determine their mitodepressive activity.

This report describes the synthesis of some sodium aminoalkanesulfonates (I–V) in which *m*- and *p*-chloroanilines were used with the corresponding aldehyde sodium bisulfites (Scheme I and Table I). In some cases, the desired products were obtained by the addition of sodium metabisulfite to azomethine derivatives (Scheme II).

Nitrosation of I–V and sodium *p*-chloroanilinophenyl-

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