(7) H. Ehrsson and B. Mellström, *Acta Pharm. Suecica*, **9**, 107(1972).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 3, 1974, from *AB KABI, Research Department, Analytical Chemistry, S-104 25 Stockholm, Sweden.*

Accepted for publication August 9, 1974.

The authors thank Gunnar Hanshoff for the synthesis of 6-methylnicotinamide.

[x] To whom inquiries should be directed.

Liquid Chromatography of Clindamycin 2-Phosphate on Triethylaminoethyl Cellulose

WALTER MOROZOWICH [x] and ROGER G. WILLIAMS

Abstract □ The separation of clindamycin 2-phosphate from clindamycin 3-phosphate, clindamycin 4-phosphate, clindamycin B 2-phosphate, and lincomycin 2-phosphate was achieved by liquid chromatography on triethylaminoethyl cellulose using a 254-nm monitor. The compounds have low molar absorptivities at 254 nm (<17), and UV detection is made possible by the high capacity support triethylaminoethyl cellulose. Linear peak height response *versus* concentration allows rapid quantitation of clindamycin 2-phosphate.

Keyphrases □ Clindamycin 2-phosphate—separation from other clindamycin compounds, triethylaminoethyl cellulose column, liquid chromatography □ Triethylaminoethyl cellulose—used for liquid chromatographic separation of clindamycin 2-phosphate from other clindamycin compounds □ Liquid chromatography—separation, clindamycin 2-phosphate from other clindamycin compounds

Clindamycin 2-phosphate[1] (clindamycin phosphate) (I) is an antibacterial agent synthesized in an attempt to decrease the pain on injection associated with the compound clindamycin (1). The purpose of this work was to explore the separation of clindamycin 2-phosphate from related isomeric phosphate esters using background absorbance monitoring (254 nm), made possible by the high capacity support triethylaminoethyl cellulose.

EXPERIMENTAL

A liquid chromatograph[2] with a 254-nm detector was used.

Column Packing—Triethylaminoethyl cellulose[3] was freed of fines by slurrying 1–4% suspensions in water and allowing the material to settle for 1–1.5 hr. The supernate was discarded and the slurrying–settling process was repeated six times. The purified support was isolated by filtration, washed with acetone, and air dried for 24 hr.

A funnel was attached to a 2.1-mm i.d. × 1-m stainless steel column, and 5–10 mg of triethylaminoethyl cellulose was added from an inverted container supported on a 50-mesh screen by brief mechanical vibration of the screen. The side of the column was vibrated for a few seconds, and the material within was compacted with a loosely fitting stainless steel rod (1.8-mm diameter) with an applied hand force of 0.2–0.7 kg (0.5–1.5 lb). The tamping rod was turned 120°, and three such compressions were made after each addition of support. Excessive compaction force results in columns with poor flow rates, and practice is required to produce columns with adequate flow (0.4–1.5 ml/min) using 2000 psi at room temperature.

The fully packed column was converted to the borate form by pumping about 50 ml of aqueous 0.5 *M* sodium borate (pH 8.80) through the column prior to use.

Mobile Phase—A solution of 0.25 *M* aqueous boric acid was prepared with addition of 10 *N* sodium hydroxide to give a pH of 8.80 ± 0.01. The solution was degassed externally for 10 min with a water aspirator while stirring vigorously.

Samples—Solutions of the phosphate esters were prepared at a concentration of 2–15% in water with addition of 5 *N* sodium hydroxide to give a pH of 8.80 ± 0.10. Injections of 7 μl were normally made using a 10-μl syringe[4] under continuous flow conditions.

Peak Identification—The identity of the collected peaks was confirmed by TLC, using silica gel plates previously sprayed with a 5% solution of boric acid, pH 8.0 (sodium hydroxide), and air dried overnight. The developing system was chloroform–methanol–water–ammonium hydroxide (40:50:6:5), and detection was achieved with a 15% ammonium sulfate spray followed by heat. The R_f values were: clindamycin 2-phosphate, 0.2; clindamycin 3-phosphate, 0.4; and clindamycin 4-phosphate, 0.3.

Phosphatase Hydrolysis (2)—A solution of 0.05–1 mg of the

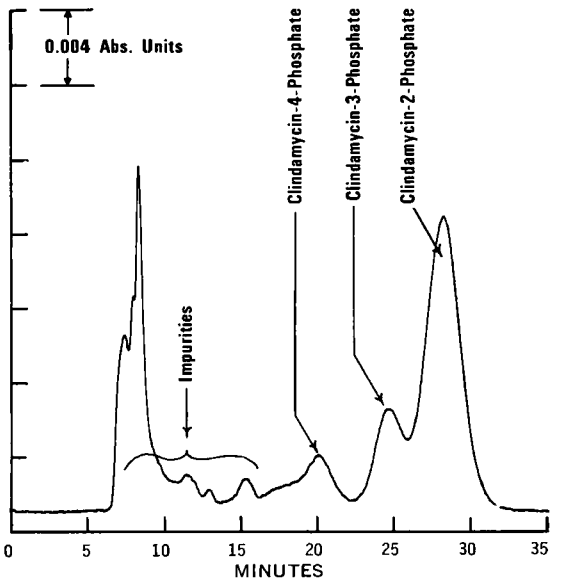
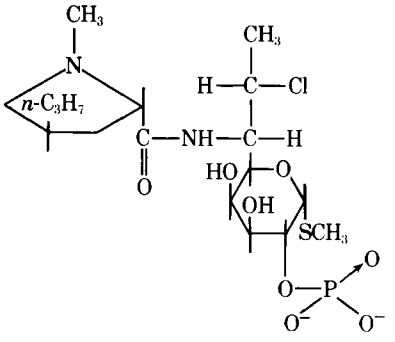


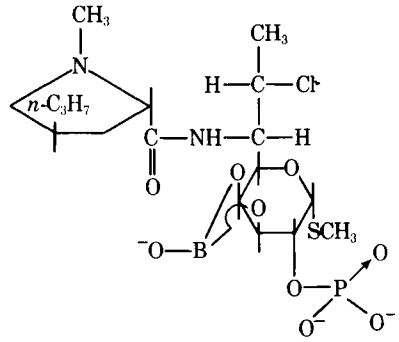
Figure 1—*Separation of about 0.4 mg of clindamycin 2-phosphate from a crude sample of the corresponding 3- and 4-phosphate esters, using a 2.1-mm × 1-m column of triethylaminoethyl cellulose and 0.25* M *boric acid (pH 8.80) at 60° (flow 0.23 ml/min, 410 psi).*

[1] Cleocin Phosphate, The Upjohn Co.
[2] Du Pont model 820.
[3] Cellex-T, BioRad Laboratories, Richmond, Calif.
[4] Hamilton 701N, Chaney type II adaptor.

reactive *cis*-3,4-diol:

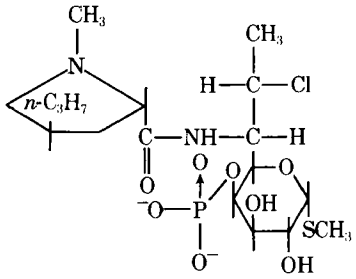


I: clindamycin 2-phosphate



II: clindamycin 2-phosphate–borate complex

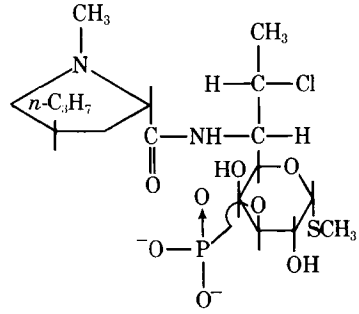
nonreactive *trans*-2,3-diol:



III: clindamycin 4-phosphate

+

nonreactive 2,4-diol:



IV: clindamycin 3-phosphate

Scheme I—Complexation of isomeric clindamycin phosphate esters with borate

clindamycin phosphate ester in 3 ml of 0.25 *M* boric acid (pH 8.8) was treated with 1 ml of 0.1 *M* magnesium chloride. Alkaline phosphatase[5], 50 mg, 1.45 units/g, was added and the mixture was vigorously shaken in a 15-ml centrifuge tube until solution was attained. After 18 hr at 37 ± 2°, the solution was shaken vigorously with 8 ml of chloroform. The mixture was centrifuged at 20,000 rpm for 30 min and the aqueous layer was removed by aspiration.

The clear chloroform layer was dried with sodium sulfate, and a 3-ml aliquot was taken to dryness under a stream of nitrogen. Trifluoroacetic anhydride (0.1 ml) was added and the vial was sealed with a Teflon-faced screw cap. The solution then was heated at 45° for 1 hr. After cooling to room temperature, about 10 mg of anhydrous sodium carbonate was added. The container was resealed immediately, and 1-μl samples were taken by syringe penetration through the Teflon-lined septum. GLC was conducted on a gas chromatograph[6] (flame-ionization detector), using a 0.6-cm × 0.6-m (0.25-in. × 2-ft) glass column containing 1% SE-30 on Gas Chrom Q (100–120 mesh) at 170°.

Crude Clindamycin 3-Phosphate and Clindamycin 4-Phosphate—A solution of 98.5 g of clindamycin 2-palmitate hydrochloride (3) in a mixture of 145 ml of pyridine and 290 ml of acetone was added dropwise to a solution of 14.3 ml of phosphorus oxychloride in a mixture of 70 ml of pyridine and 70 ml of acetone. After 0.5 hr, 600 g of ice was added and the solvent was removed *in vacuo*.

The residue was dissolved in a mixture of methanol, ether, water, and ammonium hydroxide (280 ml of each). After 20 hr at room temperature, the solution was extracted with ether and the aqueous phase was taken to dryness under vacuum. The residue was chromatographed on 190 g of silica gel[7] (0.05–0.2 mm), using chloroform–methanol–water–ammonium hydroxide (8:8:1:1). The pooled product fraction was evaporated to dryness and the residue was dissolved in methanol (100 ml). The solution was desalted by careful acidification with sulfuric acid.

After filtration, removal of the solvent gave 3.5 g of a crude mixture containing clindamycin 3-phosphate and clindamycin 4-phosphate (R_f 0.4 and 0.3, respectively, on borate–silica gel plates). The R_f values were identical with those of the authentic 3-phosphate reported previously (2) and the authentic 4-phosphate prepared by a modified route. GLC–mass spectral[8] analysis, after conversion of authentic clindamycin 4-phosphate to the tris(trimethylsilyl) derivative (1% OV-1 on Gas Chrom Q, retention time of 2.8 min at 210°), gave the following key fragmentation: *m/e* 792 (M^+), 777

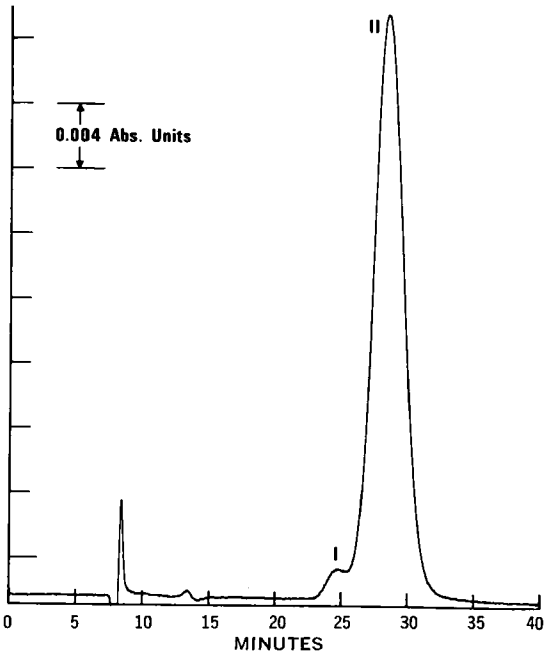


Figure 2—*Liquid chromatogram of 1.05 mg of pure clindamycin 2-phosphate (7 μl of a 15% solution, pH 8.85). Conditions were the same as in Fig. 1.*

[5] Worthington Biochemicals.
[6] Hewlett-Packard model 402-B.
[7] Merck.
[8] LKB 9000.

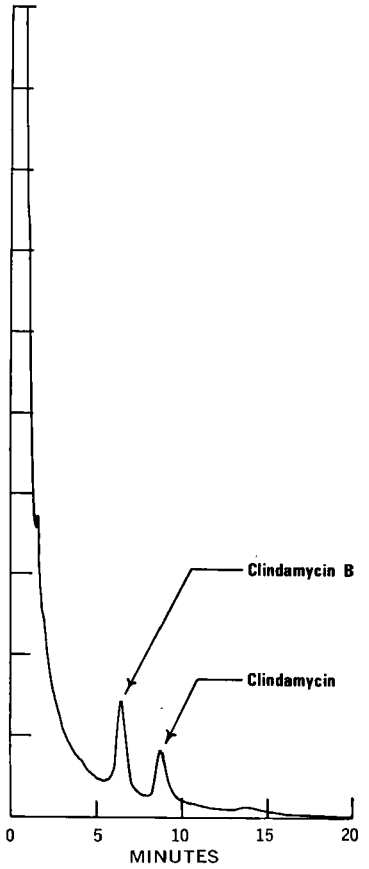


Figure 3—*Gas chromatogram of peak I from Fig. 2 after phosphatase hydrolysis and conversion to the trifluoroacetate derivative (conditions: 1% SE-30, 170°).*

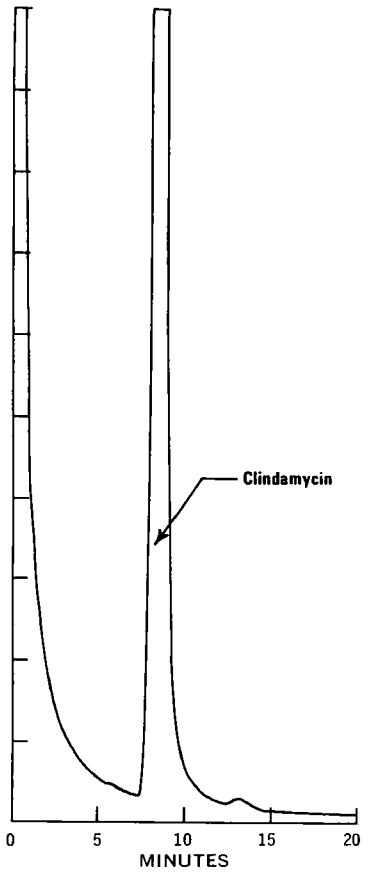


Figure 4—*GLC of peak II from Fig. 2 after phosphatase hydrolysis and conversion to the trifluoroacetate derivative. Conditions were the same as in Fig. 3.*

($M^+ - CH_3$), 756 ($M^+ - HCl$), 741 ($M^+ - HCl - CH_3$), and 713 ($M^+ - HCl - C_3H_7$).

Phosphate analysis, according to the method of Bartlett and Lewis (4), showed a phosphate content of 5.25% for authentic clindamycin 4-phosphate, which is in agreement with the theoretical value of 5.76%.

RESULTS

Borate was chosen as the mobile phase for liquid chromatographic studies since reversible interaction should occur with clindamycin 2-phosphate (I) but not with clindamycin 3-phosphate (IV) or clindamycin 4-phosphate (III) (Scheme I). Borate interaction is specific for vicinal *cis*-diols (5) and is provided only with the 2-phosphate ester.

Liquid chromatography was conducted using a 254-nm detector; the conditions for chromatography were: 0.25 *M* boric acid, pH 8.80, 410 psi, 0.23 ml/min at 60°. The isomeric phosphate esters were eluted in the order clindamycin 4-phosphate < clindamycin 3-phosphate < clindamycin 2-phosphate (Fig. 1), as verified by TLC analysis of the collected peaks.

As expected, clindamycin 2-phosphate was eluted last, presumably due to the retarding influence of the increased anionic character of the borate complex II. The separation of the 2- and 3-phosphate esters in the borate system was superior to that provided by several other electrolytes. Lincomycin 2-phosphate (6) is well separated from clindamycin 2-phosphate (retention times of 21.7 and 28.2 min, respectively).

The chromatograms of pure clindamycin 2-phosphate show the presence of a minor peak preceding the major peak (Fig. 2). Peak collection followed by phosphatase hydrolysis (2) and GLC analysis in comparison with authentic standards showed that the major liquid chromatographic peak II (Fig. 2) was clindamycin 2-phosphate and the minor liquid chromatographic peak I was clindamycin B 2-phosphate. Phosphatase hydrolysis liberated clindamycin from the former and clindamycin B from the latter, as established by GLC (Figs. 3 and 4) in comparison to retention times with authentic samples (7). Clindamycin B is the 4′-ethyl analog of clindamycin, which is produced in the synthesis of clindamycin (8) from the biosynthetically prepared lincomycin containing lincomycin B (9).

The theoretical plate number (10) for the clindamycin 2-phosphate peak (Fig. 2) showed that approximately 750 theoretical plates were attained on the 1-m triethylaminoethyl cellulose column. The column is remarkably stable, and one column was used for 3 months at 60° with little change in retention times or theo-

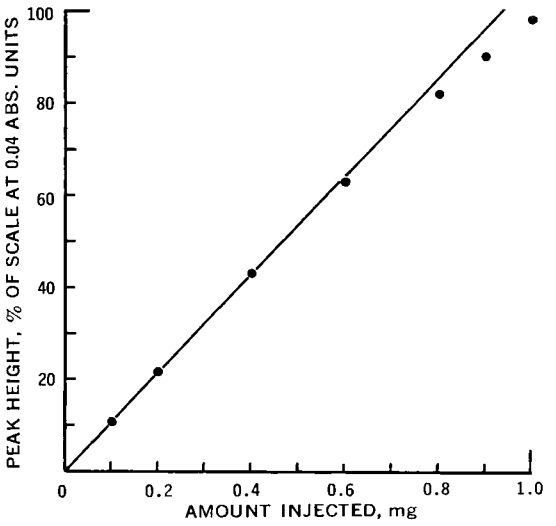


Figure 5—*Liquid chromatographic peak height response* versus *amount of clindamycin 2-phosphate injected.*

retical plate number. At room temperature, but not at 60°, a progressive decrease in flow rate is often experienced.

Plots of peak height *versus* amount of clindamycin 2-phosphate injected showed slight deviation from linearity at high sample charges (0.8–1.0 mg) (Fig. 5). Below 0.6 mg, excellent linearity is observed and peak heights can be used for quantitation.

DISCUSSION

The separation of clindamycin 2-phosphate from isomeric and related phosphate esters is sufficient to allow quantitation by the peak height approach.

Detection of clindamycin 2-phosphate by UV monitoring of background absorbance is remarkable and worthy of comment. The molar absorptivity of clindamycin 2-phosphate at 254 nm is only 16.8 in water at pH 8.90. Detection of clindamycin phosphate was made possible only by use of the high capacity support triethylaminoethyl cellulose. The exchange capacity of a 2.1-mm × 1-m column is 810–1160 μEq, as calculated from the column weight and the exchange capacity of the support. This value is approximately 20 times larger than the conventional liquid chromatographic ion-exchange supports which normally contain 48–60 μEq in the same size column (11). The use of high capacity supports should allow the detection of many compounds with low background absorbance at 254 nm.

REFERENCES

(1) W. Morozowich and D. J. Lamb, U.S. pat. 3,487,068 (1969).

(2) T. F. Brodasky and C. Lewis, *J. Antibiot.*, **1972**, 2651.

(3) W. Morozowich and A. A. Sinkula, U.S. pat. 3,580,904 (1971).

(4) E. M. Bartlett and D. H. Lewis, *Anal. Biochem.*, **36**, 159(1970).

(5) R. J. Ferrier and W. G. Overend, *Quart. Rev.*, **13**, 265 (1959).

(6) W. Morozowich, D. J. Lamb, F. A. MacKellar, C. Lewis, K. F. Stern, and E. L. Rowe, *J. Pharm. Sci.*, **58**, 1485(1969).

(7) B. J. Magerlein, R. D. Birkenmeyer, and F. Kagan, *J. Med. Chem.*, **10**, 355(1967).

(8) R. D. Birkenmeyer and F. Kagan, *ibid.*, **13**, 616(1970).

(9) R. L. Houtman, A. J. Taraszka, and D. G. Kaiser, *J. Pharm. Sci.*, **57**, 693(1968).

(10) B. L. Karger, in "Modern Practice of Liquid Chromatography," J. J. Kirkland, Ed., Wiley-Interscience, New York, N.Y., 1971, p. 3.

(11) J. J. Kirkland, *J. Chromatogr. Sci.*, **8**, 72(1970).

ACKNOWLEDGMENTS AND ADDRESSES

Received June 28, 1973, from the *Research Laboratories, The Upjohn Company, Kalamazoo, MI 49001*

Accepted for publication August 28, 1974.

The technical assistance of Mr. Scott Douglas is appreciated.

[x] To whom inquiries should be directed.

Simultaneous High-Speed Liquid Chromatographic Determination of Tetracycline and Rolitetracycline in Rolitetracycline Formulations

A. G. BUTTERFIELD [x], D. W. HUGHES, W. L. WILSON, and N. J. POUND

Abstract □ A rapid, precise high-speed liquid chromatographic procedure for the simultaneous determination of tetracycline and rolitetracycline in rolitetracycline formulations is described. Samples are dissolved in water, chilled to 0°, and chromatographed on a pellicular cation-exchange resin. The specificity of this method represents a significant improvement over present analytical procedures, which fail to differentiate between rolitetracycline and its hydrolysis product, tetracycline, in these formulations.

Keyphrases □ Rolitetracycline and rolitetracycline nitrate formulations—simultaneous high-speed liquid chromatographic analysis of tetracycline and rolitetracycline □ Tetracycline and rolitetracycline in rolitetracycline and rolitetracycline nitrate formulations—simultaneous high-speed liquid chromatographic analysis □ High-speed liquid chromatography—analysis, simultaneous, rolitetracycline and tetracycline in rolitetracycline and rolitetracycline nitrate formulations

Rolitetracycline (I) (2-*N*-pyrrolidinomethyltetracycline) is an antibiotic formed by *N*-aminomethylation of the carboxamide function of tetracycline (II). Compound I is formulated either as the base or nitrate salt for intravenous or intramuscular use. These formulations are intended to be made at the time of use with water suitable for injection.

The official method of assay of I in Canada (1) is microbiological. This method, however, gives no information on the extent of hydrolysis of I, since the test organism is also sensitive to II, nor any estimate of epimerization or degradation products expected to be formed under the assay conditions used. One would expect I, a Mannich base, to be susceptible to hydrolysis in aqueous solution, and Brunzell (2) demonstrated that aqueous solutions of I are indeed rapidly hydrolyzed with the formation of II and 4-epitetracycline (III).

By using high-speed liquid chromatography (HSLC), the stability of I was studied and it was confirmed that aqueous solutions are rapidly hydrolyzed to II (3). In addition, formulations of I were also found to contain II. Although the hydrolysis of I to II does not involve a loss of microbiological activity, the presence of II in formulations could be an indication of poor manufacturing practice.

This report describes a rapid HSLC procedure for the simultaneous assay of I and II in rolitetracycline formulations. Being precise and highly specific, the method represents a significant improvement over present analytical procedures for the quality control of rolitetracycline formulations.