Tetracycline Epimerization Kinetics Utilizing NMR Spectrometry

K. D. SCHLECHT▲ and C. W. FRANK

Abstract □ NMR was used to monitor the epimerization of tetracycline since the dimethylamino resonance of tetracycline and its C-4 epimer differ by 0.1 p.p.m. This method eliminated the need for acidification in the previously reported spectrophotometric method, thereby lessening the possibility of anhydro formation which occurs in the presence of mineral acids. Anhydro formation would be readily detected *via* the NMR method.

Keyphrases □ Tetracycline epimerization—kinetics, monitored using NMR □ Epimerization, tetracycline—kinetics, monitored using NMR □ NMR spectroscopy—monitoring tetracycline epimerization kinetics

Tetracyclines were first reported to epimerize in 1955 when Doerschuk *et al.* (1) reported a reversible "isomerization" for chlortetracycline, bromotetracycline, oxytetracycline, and tetracycline[1] (I). The epimer of I was named 4-epitetracycline (II) when the reaction was shown to take place at the C-4 position (2, 3). McCormick *et al.* (4) reported that epimerization occurred in aqueous solutions of pH 2–6 in the presence of anions such as phosphate, acetate, and citrate. The reactions reached equilibrium when 20–55% of the epimer was produced. The epimers were found to have substantially lower antibiotic activity against a large variety of tetracycline-susceptible microorganisms.

In 1963, Remmers *et al.* (5) reported additional kinetic data on the epimerization in the presence of phosphate and citrate. In 1968, Hussar *et al.* (6) published the results of their studies in the presence of acetate. Both these reports made use of a spectrophotometric absorbance ratio technique very similar to the one first reported by McCormick *et al.* (4). The technique is based on the difference in molar absorptivity (ϵ) of I (approximately 19,000) and II (approximately 15,000) at 267 nm., as well as the presence of an isosbestic point at 254 nm. Aliquots of the reaction mixtures are acidified and then absorbances are measured. The results of some previous work (7) indicate that small amounts of anhydrotetracycline (III) may form when solutions of I are acidified. Compound III has a very high ϵ (approximately 40,000) at 272 nm., so even small amounts of III could seriously affect the spectrophotometric method of assay.

NMR spectra of various tetracyclines were obtained and studied to determine if NMR would be a useful method of monitoring the kinetics of epimerization. Von Wittenau and Blackwood (8) first reported a difference in the chemical shifts of the dimethylamino group in I and II of about 0.1 p.p.m. in pyridine solvent. The NMR method could eliminate the need for acidification of reaction aliquots. Small amounts of III should be easily detected due to its significantly different structure, yet this should not significantly influence the kinetic monitoring.

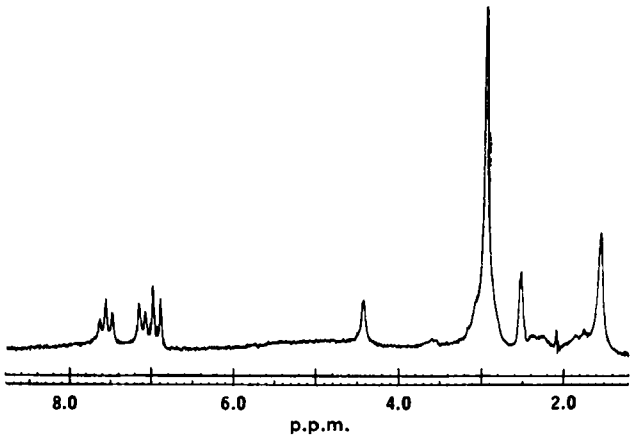

Figure 1—*NMR spectrum of tetracycline hydrochloride in dimethyl sulfoxide-*d_6.

EXPERIMENTAL

The I-HCl and dedimethylaminotetracycline (IV) were obtained from a commercial source[2]. Compound II was prepared by a method very similar to that reported by McCormick *et al.* (4); 4.0 g. of I-HCl was dissolved in isopropanol–methanol–hydrochloric acid (4:1:2), heated to 70° for at least 0.5 hr., cooled, and filtered. Recrystallization from methanol–hydrochloric acid (30:1) yielded yellow needlelike crystals which were dried under vacuum for 2 days, yielding 1.91 g. of crystals (m.p. 219–222° dec.). The product was found to be pure by TLC. The TLC method used acid-washed Kieselguhr (MN) slurried with 5% calcium disodium edetate (EDTA) at pH 7.5, as reported by Fernandez *et al.*(9). Compound I (base) was prepared by dissolving I-HCl in water near saturation and

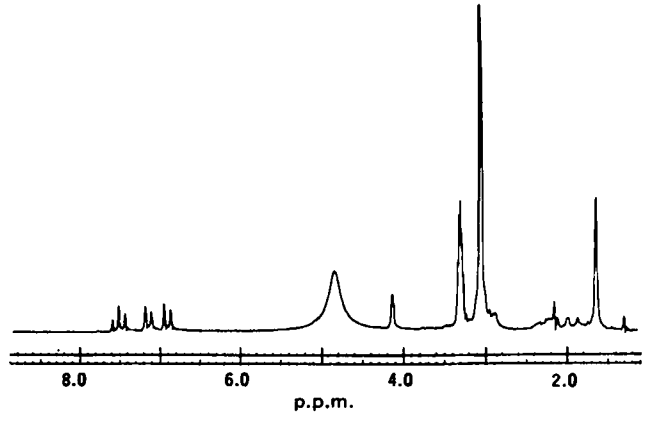

Figure 2—*NMR spectrum of tetracycline hydrochloride in methanol-*d_4.

[1] 4-(Dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide.

[2] Chas. Pfizer and Co.

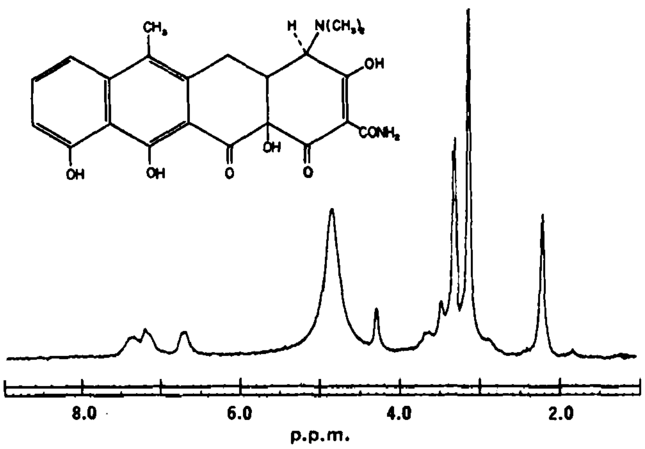

Figure 3—*NMR spectrum of tetracycline (base) in acetone-*d_6.

then adjusting to pH 5.5 with aqueous ammonia. The resulting precipitate was filtered, washed three times with water, and dried under vacuum at 60° (m.p. 175° dec.).

NMR spectra were obtained on a spectrometer[3]. Deuterated solvents were obtained from a commercial source[4]. Tetramethylsilane was used as an internal reference and lock. The solvent used for epimerization studies was a mixture of methanol-d_4 and D_2O, approximately 0.5 *M* in sodium dihydrogen phosphate. More specifically, 1 *M* NaH_2PO_4 was prepared in D_2O and then 1 ml. of this solution was mixed with 0.884 g. methanol-d_4. Approximately 30 mg. of I-HCl was dissolved in 1 ml. of this solvent. If the solvent composition differed significantly from that described here, either the desired concentration could not be achieved or a I-phosphate complex would precipitate (2). The apparent pH's of the reaction mixtures were determined using a semimicro combination electrode[5] and a pH meter[6] standardized with pH 4.00 buffer. They ranged from 4.05 to 4.10.

The NMR spectra during epimerization were obtained and integrated on the spectrometer. Tetramethylsilane sealed in a drawnout melting-point capillary was used as an external lock signal. Reproducible lock signals were obtained with such an external reference; however, each sealed capillary provided different lock signals relative to each other. The chemical shifts of benzene and chloroform were determined with each tetramethylsilane capillary, and each was corrected to the literature values for these compounds.

Prepared reaction mixtures were placed in an NMR sample tube and left in the probe of the instrument until equilibrium was approached. The temperature was maintained by a variable temperature probe unit[7]. The actual temperature was determined by measuring the difference in chemical shifts of the hydroxyl and methylene protons in ethylene glycol. Recent literature (10–12) indicated

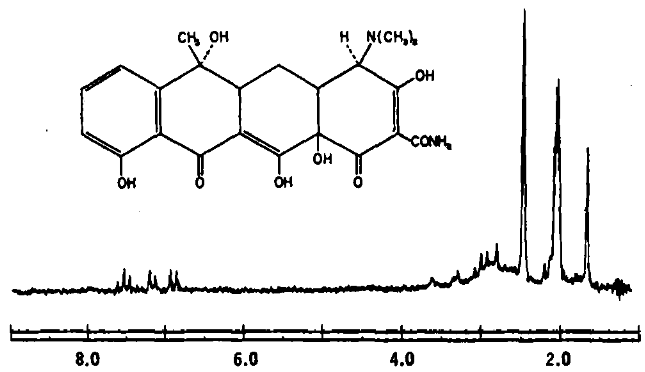

Figure 4—*NMR spectrum of anhydrotetracycline in methanol-*d_4.

[3] Varian HA-100 spectrometer.
[4] Stohler Isotopes.
[5] Corning.
[6] Fisher Accumet model 210.
[7] Varian.

Table I—Chemical Shifts (p.p.m.) of Some Protons of Tetracyclines

Compound	Solvent	CH_3 (C-6)	$N(CH_3)_2$ (C-4)	H (C-7)	H (C-8)	H (C-9)
I-HCl	Dimethyl sulfoxide-d_6	1.55	2.94	7.11	7.55	6.93
I-HCl	Methanol-d_4	1.62	3.05	7.13	7.50	6.89
I (base)	Acetone-d_6	1.67	2.49	7.14	7.52	6.88
IV	Acetone-d_6	1.62	—	7.13	7.51	6.88

Table II—Chemical Shifts (p.p.m.) of Anhydrotetracycline Hydrochloride (III)

Relative Concentration	CH_3 (C-6)	$N(CH_3)_2$ (C-4)
1	2.23	3.15
One-half	2.33	3.11
One-eighth	2.42	3.05

some discrepancy in the [illegible] equat[illegible] that have [illegible] (10) for converting the temperature was used:

$$T°K. = 411.0 - 1.694\ \Delta c.p.s.\ (\text{for 60-MHz. values}) \quad (Eq. 1)$$

RESULTS [illegible] DISCUSSION

The NMR spectra of I-HCl were obtained in dimethyl sulfoxide-d_6 and methanol-d_4 as shown in Figs. 1 and 2, respectively. The spectrum of I (base) was obtained in acetone-d_6 as shown in Fig. 3. The data are summarized in Table I. Assignments were made using cataloged protons of similar classification (13). The difference in the chemical shifts of the two methyl groups of the C-4 nitrogen in I-HCl and I (base) can be readily explained since the methyl groups in I (base) are attached to a tertiary nitrogen in contrast to a quaternary ammonium nitrogen. The chemical shifts of IV in acetone-

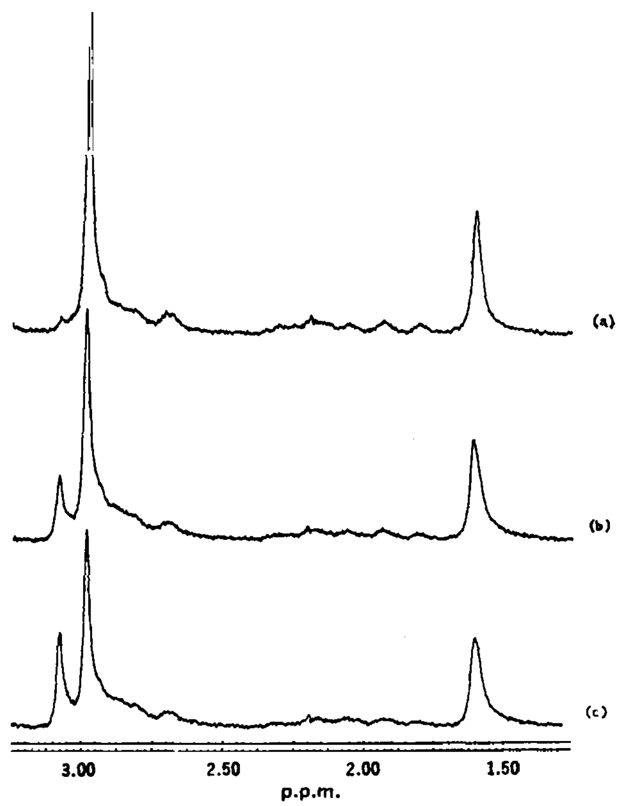

Figure 5—*NMR spectrum of tetracycline epimerization reaction mixture after* (a) *0.5,* (b) *10, and* (c) *24 hr. at 35.9°.*

Table III—Reversible First-Order Rate Constants for Epimerization of Tetracycline at Various Temperatures

Temperature	Rate Constants ($hr.^{-1}$) × 10^2		
	$k_1 + k_{-1} \pm$ Estimated *SD*	k_1	k_{-1}
52.0°	50 ± 1.1	20	30
46.2°	25 ± 0.5	10	15
35.9°	9.8 ± 0.3	3.9	5.9

d_6 are also reported in Table I. The D-ring splitting constants of all these compounds were 8 Hz.

The NMR spectrum of III-HCl in methanol-d_4 was also obtained as shown in Fig. 4. The chemical shift of the C-6 methyl appeared at approximately 2.3 p.p.m., and the two methyl groups on the C-4 nitrogen were at approximately 3.1 p.p.m. The D-ring was not well resolved; the bands were relatively broad and were not distinct doublets and triplets. It was not possible to report chemical shifts accurately for any of the protons of III since they were found to be concentration dependent. Table II lists the observed chemical shifts at various concentrations. The D-ring protons were also shifted approximately 0.3 p.p.m. downfield as the concentration was decreased, but they were the first to be lost in the background noise at low concentrations.

It is concluded that the concentration-dependent chemical shifts in III are due to an intermolecular complex, since the degree of intermolecular complexation should decrease with a decrease in concentration. Compound I was subjected to similar concentration experiments, and no significant shifts were observed. All shifts changed less than 0.05 p.p.m. over the same relative concentration range of Table II.

The NMR spectra of tetracyclines indicated that the formation of III during epimerization would be easy to detect due to the resonances of the C-6 methyl groups. Therefore, NMR was used to detect the progress of epimerization reactions in methanol-d_6–D_2O solutions in the presence of phosphate. Three typical spectra obtained at various times during a reaction are shown in Fig. 5. The C-6 methyl group signal at 1.6 p.p.m. appears to broaden slightly with time; however, this is probably due to the C-6 methyl of II which is about 1–2 Hz. upfield of the one for I. The intense signal at 2.97 p.p.m. is due to the two methyl groups of the C-4 nitrogen of I and the signal at 3.07 p.p.m. is due to the same group on II. There is no observable signal formed in the vicinity of 2.3 p.p.m., indicating no III formation.

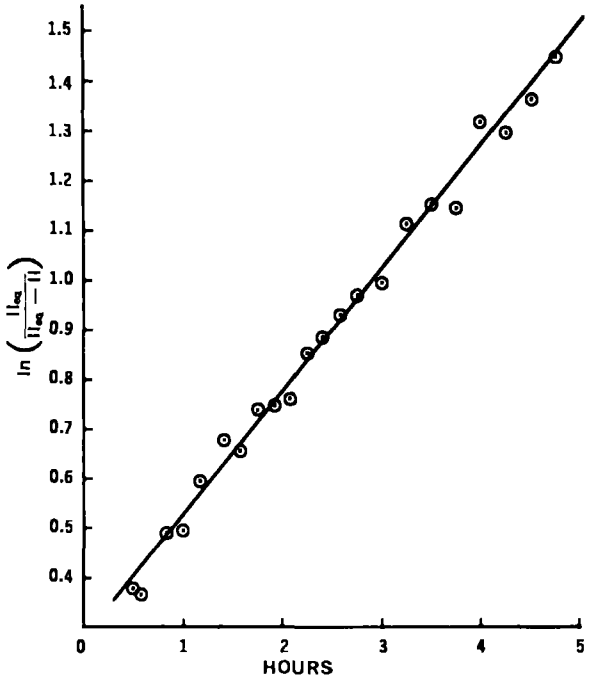

Figure 6—*Reversible first-order kinetic plot of tetracycline epimerization at 46.2°*

The various spectra were electronically integrated on the spectrometer. The area of the dimethylamino group of II was compared to the total area of the C-6 methyl groups of I and II. This would compensate for small differences in spectrum output or slight changes in tuning from spectrum to spectrum.

Reversible first-order kinetics were found to fit the data, in agreement with earlier workers (1, 4–6); a typical fit is shown in Fig. 6. A derivation of the equations used for fitting the kinetics may be found in the *Appendix*.

The equilibrium mixtures were found to contain 40% II under these experimental conditions. No significant deviations were observed with a change in temperature. This is in agreement with Remmers *et al.* (5), who reported approximately 38% epimer in 0.1 *M* phosphate at pH 4.0 in aqueous solutions. Hussar *et al.* (6) found some variation in the percent epimer at equilibrium as the temperature was varied in 0.1 *M* acetate solutions. However, in 1.0 *M* acetate, no such variations were reported.

Values obtained for k_1 and k_{-1} are listed in Table III. Interpolating the data presented by Remmers *et al.* (5) yielded reasonable correlation with these data.

An Arrhenius plot of the data in Table III yields an activation energy of 19 kcal./mole (*SD* 0.9). Remmers *et al.* (5) reported a value of 20.4 kcal./mole in 0.1 *M* phosphate at pH 4.0 in aqueous solution. Hussar *et al.* (6) also reported activation energies in aqueous solutions in this vicinity.

The values reported here using the NMR techniques compare quite favorably with the values previously reported using the absorbance ratio technique. The NMR method of monitoring the epimerization of I does not require an acidification step prior to the analysis of reaction mixtures, thereby minimizing the possibility of anhydro formation; in fact, no anhydro formation was detected. The reaction mixture can conveniently be monitored more frequently than by the previously reported methods.

APPENDIX

The following describe the first-order reversible reaction involved in the epimerization of tetracycline:

$$I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} II$$

Scheme I

$$\ln\left(\frac{II_{eq}}{II_{eq}-II}\right) = (k_1 + k_{-1})t \qquad \text{(Eq. A1}a\text{)}$$

$$\ln II_{eq} - \ln(II_{eq} - II) = (k_1 + k_{-1})t \qquad \text{(Eq. A1}b\text{)}$$

A computer program for nonreversible first-order kinetics will fit data to the following form:

$$\ln c_o - \ln c = kt \qquad \text{(Eq. A2)}$$

where c_o is a constant.

The term $\ln II_{eq}$ of Eqs. A1*a* and A1*b* is a constant like $\ln c_o$ in Eq. A2. If the term $(II_{eq} - II)$ is used in place of c in Eq. A2, the k obtained from the computer program will equal $k_1 + k_{-1}$.

The following derivation shows how to obtain k_1 and k_{-1}:

$$\frac{-d[I]}{dt} = k_1[I] - k_{-1}[II] \qquad [II] = [I]_o - [I] \qquad \text{(Eq. A3}a\text{)}$$

$$= k_1[I] - k_{-1}([I]_o - [I]) \qquad \text{(Eq. A3}b\text{)}$$

$$\frac{-d[I]}{dt} = k_1[I] - k_{-1}[I]_o + k_{-1}[I] \qquad \text{(Eq. A3}c\text{)}$$

$$= (k_1 + k_{-1})[I] - k_{-1}[I]_o \qquad \text{(Eq. A3}d\text{)}$$

$$= 0 \quad \text{at equilibrium} \qquad \text{(Eq. A3}e\text{)}$$

Therefore:

$$(k_1 + k_{-1})[I]_{eq} = k_{-1}[I]_o \qquad [I]_o = 100\%$$
$$[I]_{eq} = 100 - \%II_{eq} \qquad \text{(Eq. A4)}$$

$$\frac{k_{-1}}{(k_1 + k_{-1})} = \frac{100 - \%\Pi_{eq}}{100} \quad \text{(Eq. A5)}$$

$$k_{-1} = \frac{100 - \%\Pi_{eq}}{100}(k_1 + k_{-1}) \quad \text{(Eq. A6)}$$

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Multiple-Dose Kinetics of Oral Anticoagulants: Methods of Analysis and Optimized Dosing

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Abstract □ A mathematical technique for estimating the kinetic parameters that control patient response to oral anticoagulant administration is presented. The technique utilizes routinely obtained and recorded data such as anticoagulant dose regimen and prothrombin times. It is possible to apply during any stage of therapy and to gain predictive capability from data covering the measured response over a minimum period of 2 days. The applicability of the technique is demonstrated by direct comparison with two actual patient records.

Keyphrases □ Anticoagulant therapy—estimation of kinetic response parameters using routine clinical data, equations, compared to patient records □ Multiple-dosing kinetics, oral anticoagulants—estimation of response parameters using routine clinical data, equations, compared to patient records □ Prothrombin time data—used to estimate kinetic response parameters to oral anticoagulant therapy, equations □ Dosing regimens, oral anticoagulants—determined using estimated kinetic response parameters based on routine clinical data, equations

The pharmacological effects of hypoprothrombinemic anticoagulant drugs vary widely among individuals and preclude a universal response to a fixed dose of these agents. A given dosage schedule may be totally inadequate to prevent thrombosis in one individual but may cause hemorrhage in another (1). This fact, as well as a need sometimes to readjust therapeutic levels of activity *during* therapy (1), obviously necessitates patient individualization of dosing regimens for these drugs and clearly emphasizes the need for predictive relationships between dosage regimens and the magnitude of drug response they produce.

The first and basic steps toward such an individualization are the elucidation of the basic biochemical mechanisms involved in the synthesis of prothrombin complex activity and an assessment of the relevant intrinsic kinetics. The excellent works of other researchers (2–9) cover the whole range of development of this stage, from the pioneering level up to a complete and extensive verification of the proposed kinetic model.

Following these fundamental works, the "engineering aspects" of the process remain to be developed in detail to close the gap between laboratory (controlled) studies and health care applications and thus to arrive at a reliable and convenient aid which could be used by the doctor in prescribing anticoagulant dose regimens. This study reports the results from the authors' initial efforts in this direction. In particular, the following may be recognized as two major engineering aspects:

1. *Parameter estimation.* A recent article (10) showed how the response to a *single* dose may be utilized to evaluate all of the kinetic constants of a given patient needed to *predict* his or her future behavior. In the present study, a method is developed that meets *all conditions* for practical application by allowing implementation at *any time* after the initiation of typical hos-