of the electronic density effect measured by σ, and the X-site polarity as measured by group dipole moments. These moments are, for X = $COCH_3$, 3.00 D; CN, 4.39; and NO_2, 4.21 (21). An increase in site polarity will tend to decrease the site binding constant (20), and both K_{11b} and K_{12a} may reflect this effect superimposed on the electron density effect.

This model of complexing suggests that K_{12} values are especially useful, leading to the question: Why is K_{12} ever equal to zero (for a two-site substrate)? According to Eq. 47, K_{12} can be zero only if $a = 0$ or if one of the site binding constants (K_{gH} or K_{GH}) is zero. This leads to the following argument. If the given site assignments are correct, K_{12b} represents binding at the COO^- site (since K_{11b} mainly describes X-site binding), and it was found that $K_{12b} = 0$ for all substrates. The existence of finite K_{12a} values (substrates 8–10) means that a is finite for these acid substrates, and suggests that it will also be finite in the corresponding base substrates. It follows that K_{12b} is zero as a consequence of the site binding constant for COO^- being zero. Letting K_{gHb} represent this quantity, and K_{GHb} the site binding constant for the X-site in the conjugate base series, it follows that:

$$K_{11b} = K_{gHb} + K_{GHb} = K_{GHb} \qquad \text{(Eq. 48)}$$

i.e., K_{11b} can be identified solely with the binding at site X.

Extension to the acid series requires the assumption that K_{GHb} for binding to X in the base series is identical with K_{GHa} for binding to X in the acid series. If approximately so, then $K_{11a} = K_{gHa} + K_{GHa} = K_{gHa} + K_{GHb} = K_{gHa} + K_{11b}$, and the site binding constant K_{gHa} for the COOH site is given approximately by $K_{11a} - K_{11b}$.

With these estimates of K_{gHa} ($K_{11a} - K_{11b}$) and K_{GHa} (K_{11b}), Eq. 47 leads to estimates of a for these substrates, since:

$$K_{12a} = \frac{aK_{gHa}K_{GHa}}{K_{gHa} + K_{GHa}} = \frac{aK_{11b}(K_{11a} - K_{11b})}{K_{11a}} \qquad \text{(Eq. 49)}$$

For compound number 8, $a = 0.51$; number 9, $a = 0.38$; and number 10, $a = 0.33$. Because of the several approximations, these calculations are unlikely to be accurate, but they are reasonable in magnitude. The interpretation of complex stability data in terms of this model and Eqs. 44 and 47 seems to be a potentially useful means for describing, understanding, and perhaps predicting complex formation behavior.

REFERENCES

(1) F. Cramer, W. Saenger, and H.-Ch. Spatz, *J. Am. Chem. Soc.*, **89,** 14 (1967).

(2) K. A. Connors and J. M. Lipari, *J. Pharm. Sci.*, **65,** 379 (1976).

(3) T. Miyaji, Y. Kurono, K. Uekama, and K. Ikeda, *Chem. Pharm. Bull.*, **24,** 1155 (1976).

(4) R. I. Gelb, L. M. Schwartz, R. F. Johnson, and D. A. Laufer, *J. Am. Chem. Soc.*, **101,** 1869 (1979).

(5) K. A. Connors and T. W. Rosanske, *J. Pharm. Sci.*, **69,** 173 (1980).

(6) R. I. Gelb, L. M. Schwartz, C. T. Murray, and D. A. Laufer, *J. Am. Chem. Soc.*, **100,** 3553 (1978).

(7) R. I. Gelb, L. M. Schwartz, and D. A. Laufer, *ibid.*, **100,** 5875 (1978).

(8) A. B. Wong, Ph.D. thesis, University of Wisconsin, Madison, Wis., 1980.

(9) K. Uekama, M. Otagiri, Y. Kanie, S. Tanaka, and K. Ikeda, *Chem. Pharm. Bull.*, **23,** 1421 (1975).

(10) R. N. Mattoo, *Trans. Faraday Soc.*, **52,** 1462 (1956).

(11) P. O. Lumme, *Suomen Kemist.*, **30B,** 168 (1957).

(12) J. Johnston, *Proc. R. Soc. London*, **A78,** 82 (1906).

(13) P. A. Kramer and K. A. Connors, *Am. J. Pharm. Educ.*, **33,** 193 (1969).

(14) B. Casu and L. Ravà, *Ric. Sci.*, **36,** 733 (1966).

(15) R. L. VanEtten, J. F. Sebastian, G. A. Clowes, and M. L. Bender, *J. Am. Chem. Soc.*, **89,** 3242 (1967).

(16) E. A. Lewis and L. D. Hansen, *J. Chem. Soc., Perkin Trans.*, **II,** 2081 (1973).

(17) R. J. Bergeron, M. A. Channing, and K. A. McGovern, *J. Am. Chem. Soc.*, **100,** 2878 (1978).

(18) T. W. Rosanske, Ph.D. thesis, University of Wisconsin, Madison, Wis., 1979.

(19) R. I. Gelb, L. M. Schwartz, B. Cardelino, and D. A. Laufer, *Anal. Biochem.*, **103,** 362 (1980).

(20) T. W. Rosanske and K. A. Connors, *J. Pharm. Sci.*, **69,** 564 (1980).

(21) E. S. Gould, "Mechanism and Structure in Organic Chemistry," Holt, Rinehart and Winston, New York, N.Y., 1959, p. 62.

Reversed-Phase Ion-Pair Chromatography of Tetracycline, Tetracycline Analogs, and Their Potential Impurities

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Abstract □ Methods are presented for the separation of tetracycline, tetracycline analogs, and their potential impurities by reversed-phase ion-pair chromatography. The mobile phase consisted of a phosphate buffer with tripropylamine or *N,N*-dimethyloctylamine as counterions and acetonitrile as the organic modifier. The chromatographic properties of the tetracyclines were significantly improved by addition of the tertiary amines. The best result was obtained with *N,N*-dimethyloctylamine as the counterion, which gave good separation efficiency and peak symmetry for most of the substances studied. Addition of the tertiary amines to the mobile phase also significantly affected the capacity factors of the tetracyclines. Stability studies of the tetracyclines showed a fast degradation of chlortetracycline to isochlortetracycline and of lymecycline to tetracycline in phosphate buffer solutions of different pH. The purity of pharmaceutical preparations of tetracyclines was also investigated.

Keyphrases □ Tetracycline—potential impurities of the drug and its analogs, reversed-phase ion-pair chromatography □ High-performance liquid chromatography—tetracycline, tetracycline analogs, and their potential impurities □ Counterions—*N,N*-dimethyloctylamine, tripropylamine, in reversed-phase ion-pair chromatography of tetracycline, tetracycline analogs, and their possible impurities

The most important impurities in tetracycline and tetracycline analogs are the epimerized, dehydrated, and epimerized dehydrated forms of the tetracyclines. Thus, quatrimycin (epitetracycline), anhydrotetracycline, and epianhydrotetracycline are the most frequently found impurities in tetracycline. Epianhydrotetracycline has been reported to be toxic in humans, but the exact safety level in pharmaceutical preparations is not known. Permitted concentrations of epianhydrotetracycline and other impurities are fixed by the European Pharmacopoeia.

BACKGROUND

Various chromatographic techniques, such as TLC (1, 2) and paper chromatography (3), have been used to analyze tetracycline, but they are

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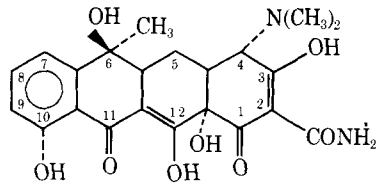

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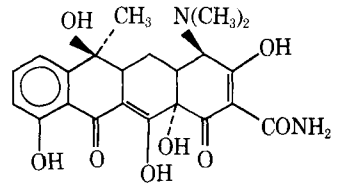

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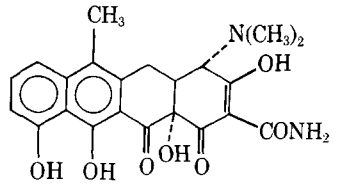

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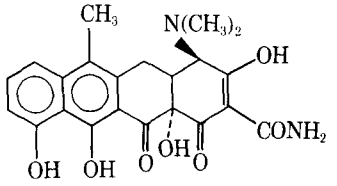

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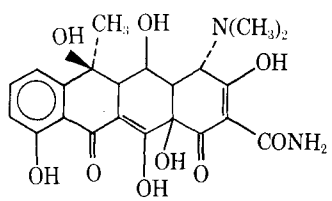
usually not sensitive enough to allow detection of small amounts of impurities with good precision.

Ion-exchange column chromatography has also been used for analysis of tetracycline and tetracycline analogs, but the separation efficiency is not good with typical plate height (H) values >5 mm (4, 5). However, better results were reported with various reversed-phase systems (6–9). The mobile phases used in these methods have pH values between 1.5 and 5. Tetracyclines were reported to undergo epimerization between pH 3 and 5, and dehydration at low pH (10–12). This means that chromatography of tetracyclines in mobile phases with pH between 1.5 and 5 is unsuitable due to possible formation of degradation products during the chromatographic run, or during the dissolution process of tetracycline preparations.

The present report describes studies of reversed-phase ion-pair chromatographic systems using mobile phases of pH 8.0. These chromatographic systems can be used for separation and quantitation of



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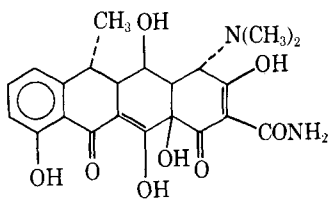

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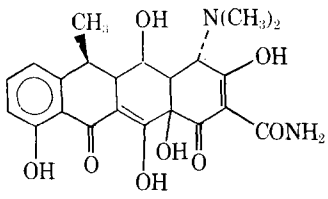

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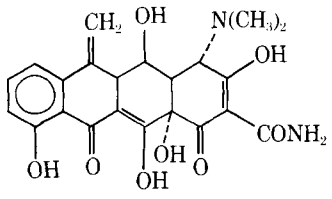

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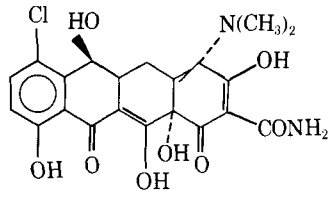

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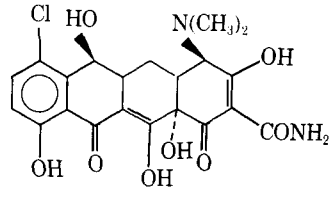

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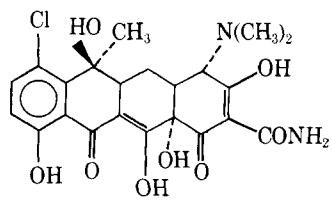

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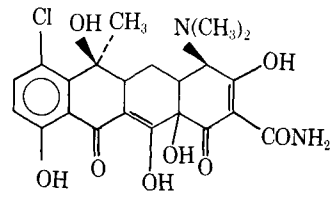


XII

tetracycline, tetracycline analogs, and related impurities. The purpose of this work was to develop chromatographic systems suitable to use in the study of small amounts of impurities (<0.1 %) in pharmaceutical preparations of tetracycline and tetracycline analogs.

EXPERIMENTAL

Apparatus—The high-performance liquid chromatographic system consisted of a high-pressure pump[1] and injection port[2]. UV detection was utilized with a detector[3] set at 280 nm. Columns (150 × 4.5-mm i.d.) were made of No. 316 stainless steel with a polished inner surface and equipped with modified end connections[4] and stainless steel frits[5] (2 μm).

Chemicals—The following chemicals were analytical grade and were used as supplied: acetonitrile[6], tripropylamine[7], and *N,N*-dimethyloctylamine[8]. All other chemicals used were analytical reagent grade and were used without further purification.

Samples of drug substances were obtained directly from the manufacturer or collected in the factory by drug inspectors. The drug preparations were bought in Swedish pharmacies. In most cases, reference samples of potential impurities were provided by the drug manufacturers. The following substances were used: tetracycline (I), quatrimycin (epitetracycline, II), anhydrotetracycline (III), epianhydrotetracycline (IV), oxytetracycline (V), doxycycline (VI), 6-epidoxycycline (VII), methacycline (VIII), demeclocycline (IX), epidemethylchlortetracycline (X), chlortetracycline (XI), epichlortetracycline (XII), isochlortetracycline (XIII), epianhydrochlortetracycline (XIV), anhydrochlortetracycline (XV), and lymecycline (XVI).

Column Packing—The columns were packed by a modification of the ordinary balanced-density slurry technique described previously (13). The support[9] was suspended in chloroform, and acetone was used as the driving liquid in the pump which was operated at 5000 psi. After packing, the column was washed with hexane followed by acetonitrile before equilibration with the mobile phase.

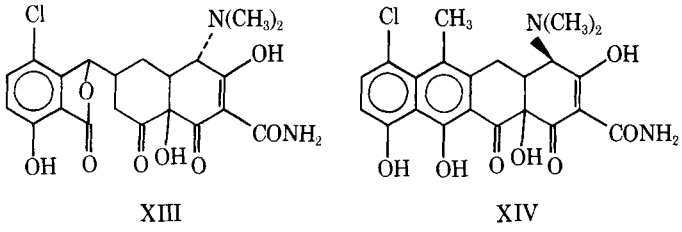
Chromatographic Technique—The chromatographic analyses were performed at room temperature. The mobile phases were prepared from 0.05 or 0.1 M monobasic sodium phosphate and the pH was adjusted to 8.2 (for the tripropylamine systems) or 8.0 (for the *N,N*-dimethyloctylamine system) with sodium hydroxide after addition of tripropylamine or *N,N*-dimethyloctylamine. An appropriate amount of acetonitrile was added and the mobile phase was thermostated at room temperature and degassed in an ultrasonic bath before use. The interstitial volume of the column, V_m, was obtained by injection of water.

Four different mobile phases were used for the purity control of the tetracyclines.

Mobile Phase A—A mixture of 0.1 M monobasic sodium phosphate, 30% acetonitrile, and 0.0194 M *N,N*-dimethyloctylamine was adjusted to pH 8.0 with sodium hydroxide.

Mobile Phase B—Mobile phase B contained 0.05 M monobasic sodium

[1] Waters model 6000, Milford, Mass.
[2] Valco model CV-UHPa 7000 psi injector equipped with a 20-μl loop, Santa Clara, Calif.
[3] Waters model 440, Milford, Mass.
[4] Crawford Fitting Co., Solon, Ohio.
[5] Altex, Berkeley, Calif.
[6] Rathburn Chemicals, Walkerburn Ltd, Peebleshire, Scotland.
[7] Fluka AG, Chemische Fabrik, Buchs, Switzerland.
[8] ICN Pharmaceuticals, Plainview, N.Y.
[9] LiChrosorb RP-8 (5 μm), E. Merck, Darmstadt, W. Germany.



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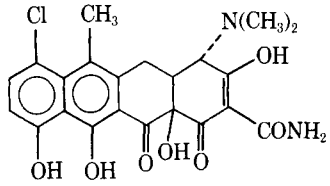

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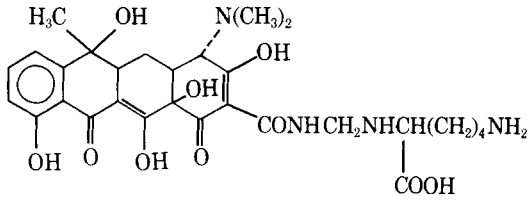

XVI

phosphate, 35% acetonitrile, and 0.0097 *M* *N,N*-dimethyloctylamine adjusted to pH 8.0 with sodium hydroxide.

Mobile Phase C—This phase contained 0.1 *M* monobasic sodium phosphate, 20% acetonitrile, and 0.0194 *M* *N,N*-dimethyloctylamine adjusted to pH 8.0 with sodium hydroxide.

Mobile Phase D—A mixture of 0.1 *M* monobasic sodium phosphate, 28% acetonitrile, and 0.0194 *M* *N,N*-dimethyloctylamine was adjusted to pH 8.0 with sodium hydroxide.

Calculation of Chromatographic Parameters—The chromatographic behavior of the tetracycline substances is expressed by the capacity factor, the plate height, and the asymmetry factor. The capacity factor, k', is calculated by:

$$k' = (t_R - t_m)/t_m \quad \text{(Eq. 1)}$$

where t_R and t_m are elution times for retained and unretained solutes, respectively. The plate height, H, is obtained from the chromatogram by:

$$H = (L/16)(w_t/t_R)^2 \quad \text{(Eq. 2)}$$

where L is the column length and w_t is the peak width at the baseline. The asymmetry factor was calculated by drawing a perpendicular to the baseline from the vertex formed by the two peak tangent lines. The back part of the peak baseline divided by the front part gives the asymmetry factor.

Standard Solutions for Chromatographic Experiments—Standard solutions were freshly prepared just before use. The substances were dissolved in mobile phases which did not contain the tertiary amine. Twenty microliters of the solutions were injected.

Tetracycline Preparations—Tablets, dragees, capsules, suspensions, and ampuls were used for this investigation. Five tablets or dragees were ground and put into a 250-ml volumetric flask. Methanol (200 ml) was added and the flask was sonicated for 10 min in an ultrasonic bath and the contents were diluted to 250 ml with methanol. An appropriate amount of this solution was then centrifuged and an aliquot was taken out and diluted with methanol to give a final concentration of 20 μg/ml (used for quantitation of active substance in the drug preparations) or 1 mg/ml (used for quantitation of impurities in the drug preparations) of active substance. One milliliter of this solution was evaporated with nitrogen at 40° and the residue was dissolved in 1.0 ml of the mobile phase (without addition of the tertiary amine). Twenty microliters of this solution were then injected on the column. The substance in the capsules was accurately weighed and dissolved in the same way and to the same concentration as for tablets and dragees. Ampuls were also handled in the same way. An aliquot of the suspensions (~10 ml) was accurately weighed and put into a volumetric flask and extracted with 100 ml of 0.1 *M* HCl in an ultrasonic bath for 10 min and finally diluted to 150 ml with

Table I—Influence of Tripropylamine Concentration on Selectivity [a]

Selectivity (α)	Tripropylamine Concentration, *M*			
	0	0.032	0.063	0.095
II				
I	2.7	3.0	2.8	2.8
IV	0.7	1.5	1.9	1.8
III	2.3	2.7	3.0	3.2

[a] Mobile phase: pH 8.2 phosphate buffer, 20% acetonitrile with varying concentrations of tripropylamine.

Table II—Influence of the *N,N*-Dimethyloctylamine Concentration on Selectivity [a]

Selectivity (α)	*N,N*-Dimethyloctylamine Concentration, *M*				
	0	0.0097	0.0194	0.0292	0.039
II					
I	2.0	2.1	2.1	1.9	1.8
IV	0.8	2.5	4.2	5.0	5.6
III	1.5	3.9	4.7	5.3	—

[a] Mobile phase: pH 8.2 phosphate buffer, 28% acetonitrile with varying concentrations of *N,N*-dimethyloctylamine.

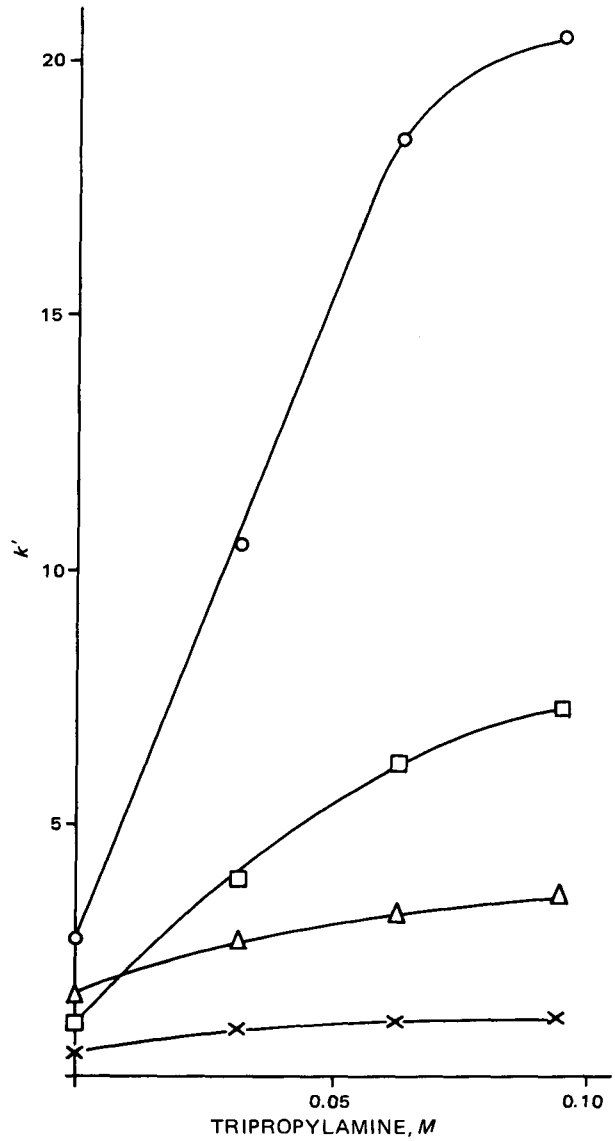

Figure 1—*Regulation of the capacity factors by addition of tripropylamine. The mobile phase consisted of pH 8.2 sodium phosphate buffer containing 20% (v/v) acetonitrile and tripropylamine at a flow rate of 1 ml/min. Key: ×, II; △, I; □, IV; and ○, III.*

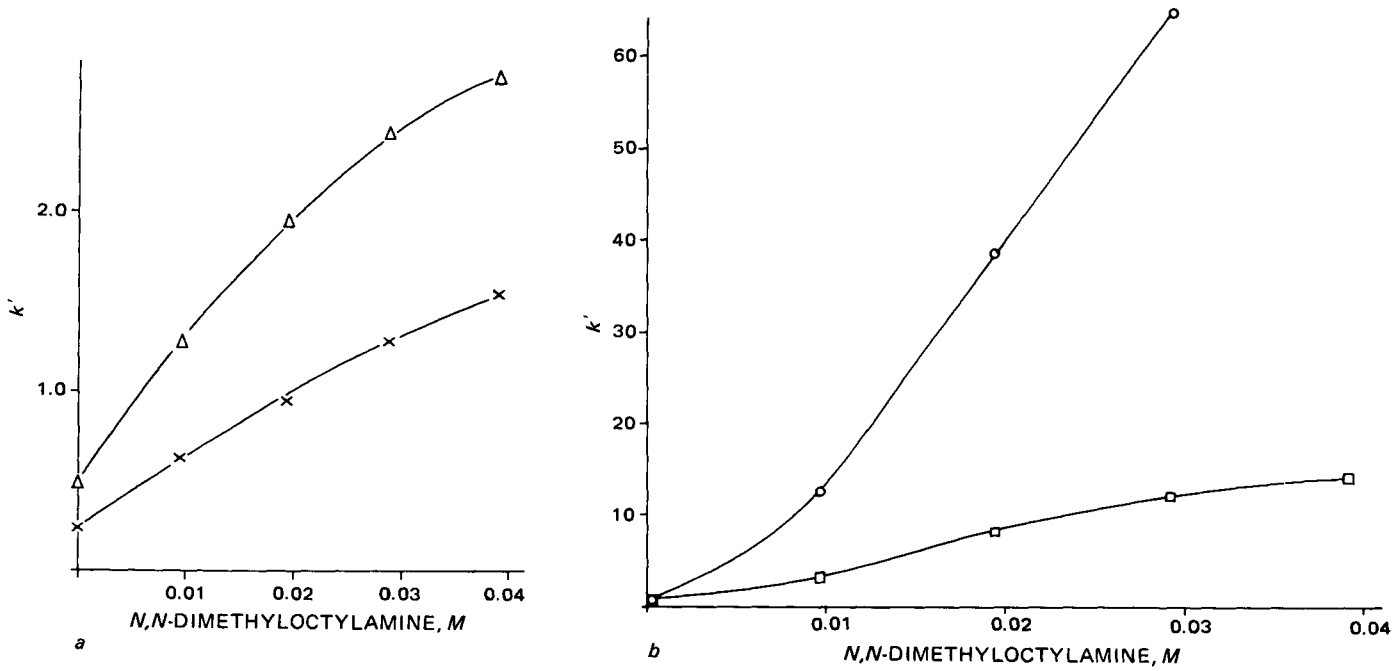

Figure 2a and 2b—*Regulation of the capacity factors by addition of* N,N-*dimethyloctylamine. The mobile phase contained sodium phosphate buffer at pH 8.2 containing 28% (v/v) acetonitrile and* N,N-*dimethyloctylamine. Key: See Fig. 1.*

0.1 *M* HCl. An aliquot of this solution was then handled in the same way as for tablets and dragees.

Drug Substances—Drug substances were dissolved as described for tetracycline preparations to a final concentration of ~1 mg/ml. A 20-μl aliquot was injected onto the column.

RESULTS AND DISCUSSION

Compounds I–IV were used as model compounds in the present study. Tetracycline has three ionization stages in the pH range of 1–12. The first pKa 3.30 involves the ionization of the acidic hydroxy group at the 3-position; the pKa values for the dimethyl amino function and the hydroxy group at position 12 are 7.68 and 9.69, respectively (14). This makes it possible to utilize ion-pair chromatography with a cationic counterion like a tertiary or quaternary amine with a mobile phase at pH 8.0 (15, 16).

Regulation of the Capacity Factor by Addition of Counter Ion—Chromatographic studies of I and its potential impurities were performed using a phosphate buffer at pH 8.2 or 8.0 and acetonitrile as organic modifier in the mobile phase. Tripropylamine and *N,N*-dimethyloctylamine were used as counterions. Twenty percent (v/v) acetonitrile was used in the tripropylamine systems and 28% in the *N,N*-dimethyloctylamine system.

One advantage of reversed-phase ion-pair chromatography is the presence of the counterion in the mobile phase, which makes it possible to regulate the capacity factor by changing the type or the concentration of the counterion. Figures 1, 2a, and 2b illustrate the regulation of the capacity factor of I, II, and the dehydrated forms III and IV by increasing the concentration of tripropylamine from zero to 0.095 *M*, and *N,N*-dimethyloctylamine from zero to 0.039 *M*. It can be seen that I and III have higher capacity factors than their corresponding epi forms, II and IV, respectively. A likely cause of this effect is that I and III have conformations which favor the formation of internal hydrogen bonding, giving the molecule a more hydrophobic character. The possibility of regulating the capacity factor of I and II is rather limited both with tripropylamine and *N,N*-dimethyloctylamine as counterions, while the capacity factors for the dehydrated substances are influenced to a large extent by the increasing concentration of counterion. The possibility of

Table III—Chromatographic Data of Tetracyclines

Compound	Mobile Phase A, k'	H, mm	Asymmetry Factor	Mobile Phase B, k'	Mobile Phase C, k'	Mobile Phase D, k'
Tetracycline (I)	1.3	0.02	1.0			
Quatrimycin (II)	0.7	0.05	1.2			
Epianhydrotetracycline (IV)	4.3	0.03	1.2			
Anhydrotetracycline (III)	17.2	0.03	1.3			
Oxytetracycline (V)	1.1	0.08	1.0	0.5		1.5
Unidentified 1	7.0					
Unidentified 2						2.1
Unidentified 3				3.3		
Doxycycline (VI)	3.4	0.07	1.0			
6-Epidoxycycline (VII)	1.6	0.18	1.7			
Demeclocycline (IX)	1.9	0.07	0.8			
Epidemethylchlortetracycline (X)	1.3	0.04	0.9		3.4	
Epianhydrodemethylchlortetracycline	9.9	0.03	1.0	2.0		
Anhydrodemethylchlortetracycline	40.5	0.04	1.0	10.0		
Unidentified					2.4	
Chlortetracycline (XI)	2.6	0.09	1.0			
Epichlortetracycline (XII)	1.5	0.40	0.2		5.0	
Isochlortetracycline (XIII)	3.9	0.05	0.8		17.0	
Epianhydrochlortetracycline (XIV)	11.1	0.04	1.0	2.2		
Anhydrochlortetracycline (XV)	48.9	0.04	0.9	6.7		
Unidentified					3.5	
Methacycline (VIII)	2.1	0.06	1.1			
Unidentified					5.9	

[a] Flow rate = 1.0 ml/min.

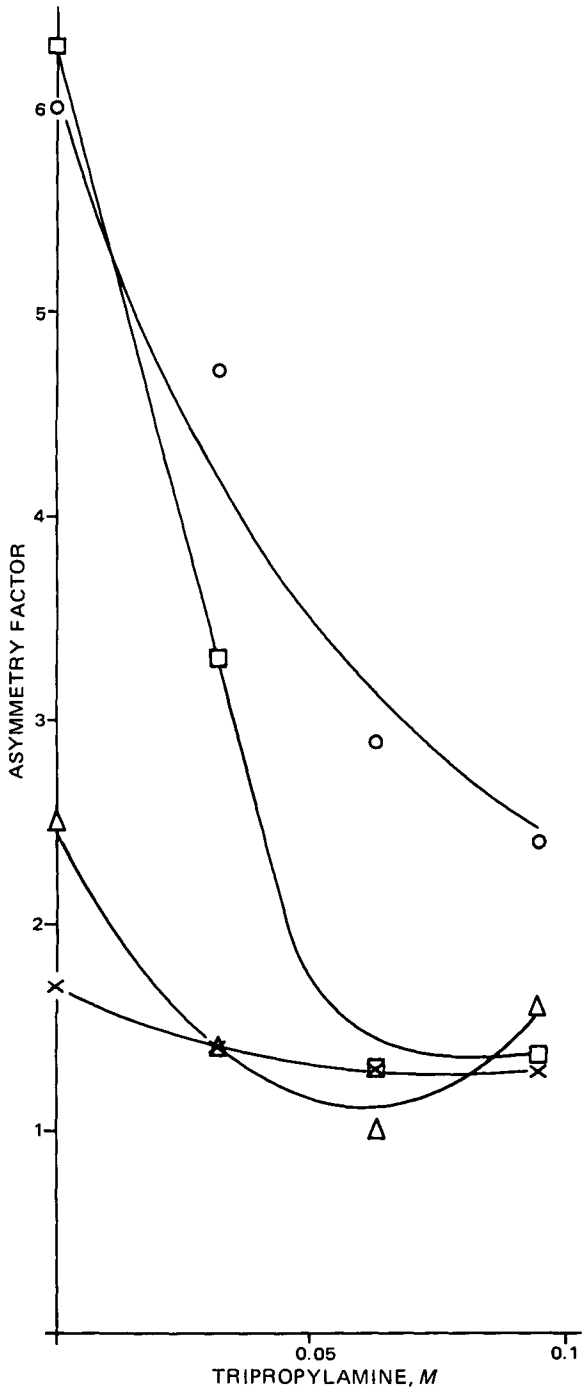

Figure 3—*Effect of the concentration of tripropylamine on peak symmetry. Key: See Fig. 1.*

regulating the capacity factors of the tetracyclines is more pronounced with *N,N*-dimethyloctylamine than with tripropylamine as counterion. This may be partly an effect of one more methylene group in *N,N*-dimethyloctylamine than in tripropylamine giving the *N,N*-dimethyloctylamine ion-pairs a more hydrophobic character.

Selectivity Change by Varying the Counterion Concentration—The influence of the nature and the concentration of the counterion on the separation factor α, is demonstrated in Tables I and II. The selectivity between II and I is better in the tripropylamine system, while the selectivity between IV and III is better in the *N,N*-dimethyloctylamine system. However, the separation factors for I and II are sufficiently large even in the *N,N*-dimethyloctylamine system to permit baseline separation at all *N,N*-dimethyloctylamine concentrations. It can also be seen that the selectivity between II and I is almost uninfluenced by an increase in the counterion concentration, while the selectivities between I and IV and also between IV and III increase with increasing counterion concentration. This effect on the separation selectivity is more

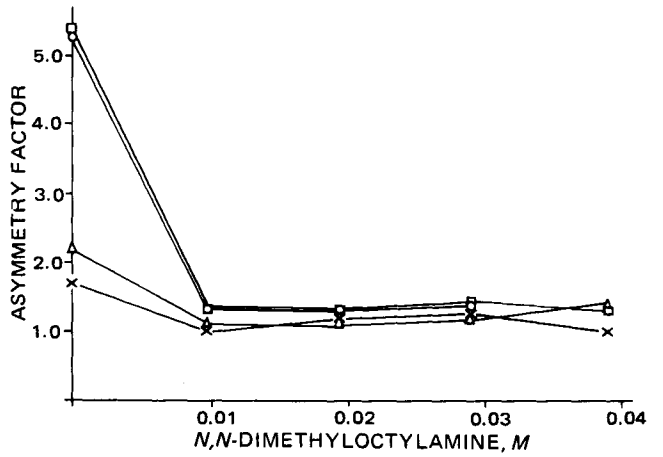

Figure 4—*Effect of the concentration of* N,N-*dimethyloctylamine on peak symmetry. Key: See Fig. 1.*

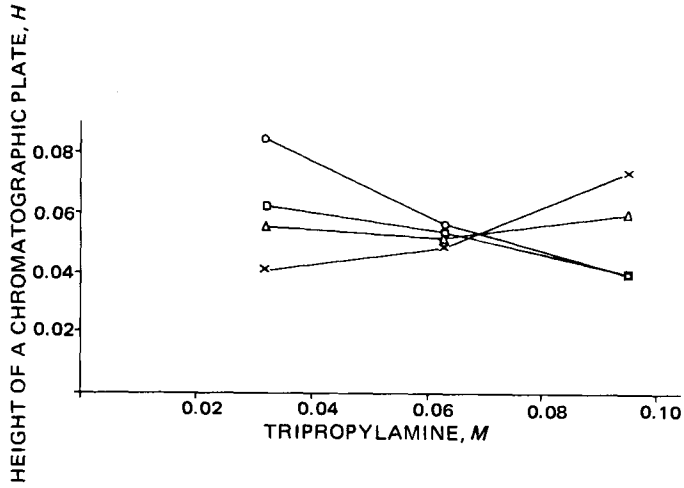

Figure 5—*Effect of the concentration of tripropylamine on separation efficiency. Key: See Fig. 1.*

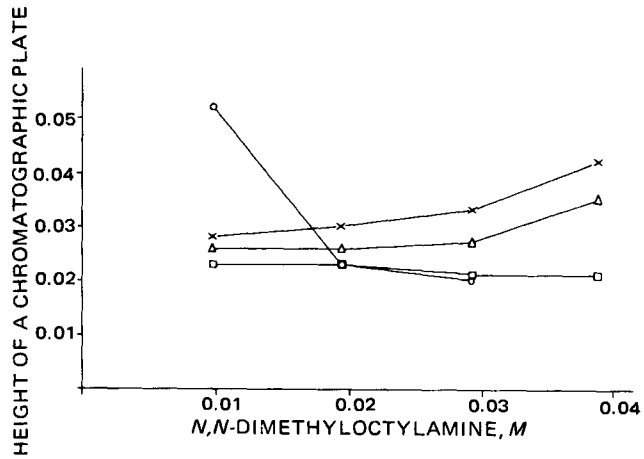

Figure 6—*Effect of the concentration of* N,N-*dimethyloctylamine on the separation efficiency. Key: See Fig. 1.*

pronounced with *N,N*-dimethyloctylamine than with tripropylamine as counterion.

Peak Symmetry and Separation Efficiency—Figure 3 demonstrates the variation in peak symmetry of the tetracyclines with increasing concentration of tripropylamine. Compounds III and IV show considerable tailing without the addition of the tertiary amine. However, tailing is significantly reduced by increasing the concentration of tripropylamine and the asymmetry factors; I, II, and IV are <2 with a tripropylamine concentration of 0.095 *M*, while III at this tripropylamine concentration still show significant tailing.

Table IV—Purity of Tetracycline Substances and Drug Preparations on the Swedish Market

Substances	Smallest Amount of Impurity, %		Largest Amount of Impurity, %		Impurity Limits in Substance, %
	Substance	Drug Preparation	Substance	Drug Preparation	Ph. Eur.
Tetracycline (I)					
Quatrimycin (II)	1.8	1.6	8.4	7.7	4.0
Chlortetracycline (XI)	n.d[a]	n.d	0.4	0.4	2.0
Epianhydrotetracycline (IV)	<0.1	n.d	0.5	0.3	0.5
Anhydrotetracycline (III)	0.1	0.2	1.9	2.2	0.5
Oxytetracycline (V)					
Unidentified 1[b]	<0.1	0.3	0.6	0.6	
Unidentified 2[b]	n.d	0.1	<0.1	0.5	
Unidentified 3[b]	n.d	<0.1	<0.1	0.2	
Doxycycline (VI)					
6-Epidoxycycline (VII)	0.2	0.1	0.9	0.6	
Methacycline (VIII)	<0.1	n.d	0.2	0.3	
Demeclocycline (IX)					
Epidemethylchlortetracycline (X)	2.0	2.1	4.0	3.8	
Epianhydrodemethylchlortetracycline	n.d.	n.d	n.d	n.d	
Anhydrodemethylchlortetracycline	n.d.	n.d	n.d	n.d	
Unidentified[c]	2.0	1.6	2.0	1.6	
Chlortetracycline (XI)					
Epichlortetracycline (XII)	n.d		n.d		
Isochlortetracycline (XIII)	~0.5		~0.5		
Epianhydrochlortetracycline (XIV)	n.d		n.d		
Anhydrochlortetracycline (XV)	0.1		0.1		
Unidentified[d]	5.2		5.2		
Methacycline (VIII)					
Unidentified[e]	0.2	0.3	0.2	0.3	

[a] n.d. = not detected. [b] Amount impurity calculated by using the molar absorptivity of V. [c] Amount impurity calculated using the molar absorptivity of epianhydrodemethylchlortetracycline. [d] Amount impurity calculated by using the molar absorptivity of XI. [e] Amount impurity calculated by using the molar absorptivity of VIII.

A much more pronounced effect on the peak symmetry of the tetracyclines was observed by the addition of *N,N*-dimethyloctylamine to the mobile phase. Figure 4 shows the variation in the asymmetry factor of I and its potential impurities with increasing *N,N*-dimethyloctylamine concentration in the mobile phase. With a *N,N*-dimethyloctylamine concentration as low as 0.01 *M*, all four tetracyclines have an asymmetry factor of <1.5. Positive effects on peak symmetry by addition of *N,N*-dimethyloctylamine were demonstrated for hydrophobic amines like imipramine, desipramine (17), and zimelidine and its metabolites (18), when chromatographed in the cationic forms. The separation efficiency of the tetracyclines is considerably improved by addition of the tertiary amines, tripropylamine, or *N,N*-dimethyloctylamine, to the mobile phase in accordance with peak symmetry. Relations between *H* and the concentration of the tertiary amines are demonstrated in Figs. 5 and 6.

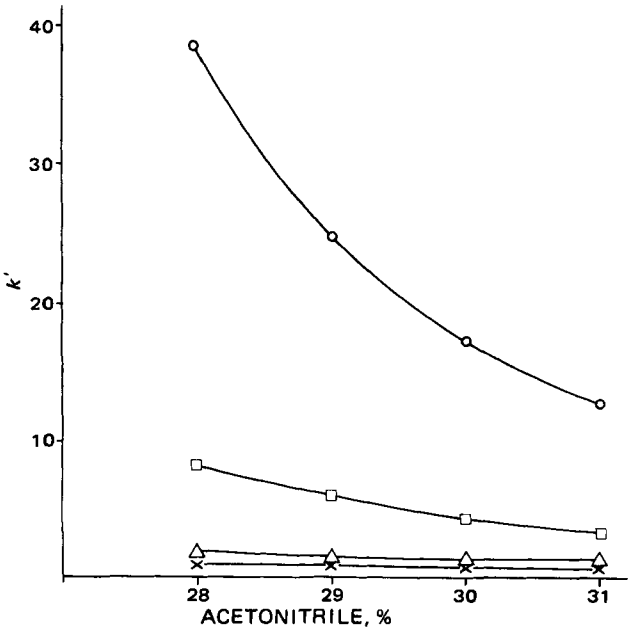

Figure 7—*Regulation of capacity factors by varying the acetonitrile concentration. The mobile phase contained a pH 8.0 sodium phosphate buffer with 0.0194* M N,N-*dimethyloctylamine and varying concentrations of acetonitrile. Key: See Fig. 1.*

Regulation of the Capacity Factor for Varying Acetonitrile Concentration—From the data presented, it follows that chromatography of the tetracyclines as *N,N*-dimethyloctylamine ion-pairs gives better chromatographic performance than when using tripropylamine as the counterion. A *N,N*-dimethyloctylamine concentration of ~0.02 *M* was sufficient to give good separation efficiency and symmetrical peaks of I–IV. Accordingly, in the following studies, the *N,N*-dimethyloctylamine system was used. From Fig. 1 it can be seen that the capacity factors of dehydrated III and IV are rather high with a *N,N*-dimethyloctylamine concentration of 0.02 *M* and 28% acetonitrile in pH 8.2 phosphate buffer.

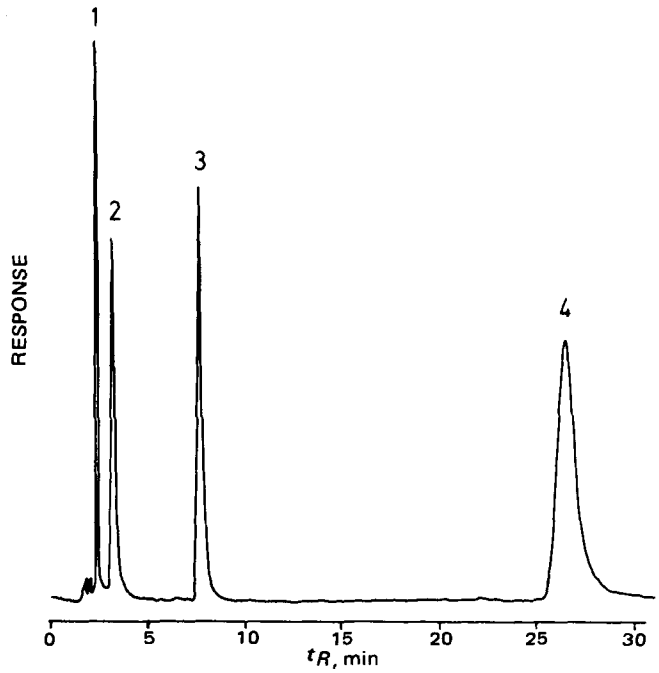

Figure 8—*Separation of I and its impurities. The mobile phase consisted of pH 8.0 sodium phosphate buffer containing 0.0194* M N,N-*dimethyloctylamine and 30% (v/v) acetonitrile at a flow rate of 1 ml/min. Key: 1, II (240 ng); 2, I (268 ng); 3, IV (246 ng); and 4, III (650 ng).*

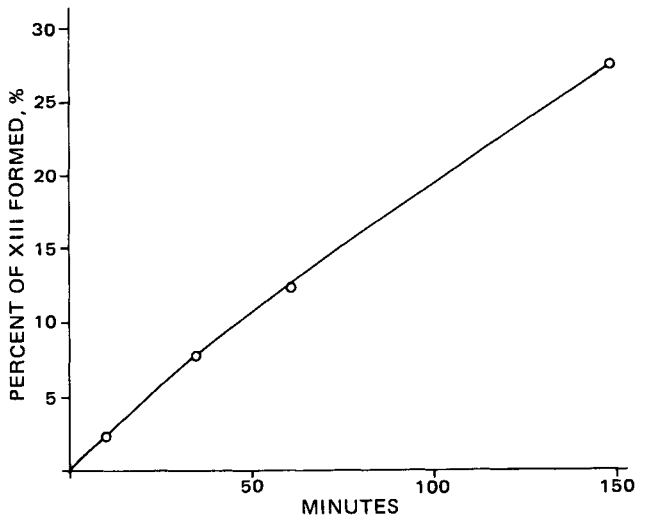

Figure 9—*Degradation rate of XI to XIII. Initial concentration of XI was 1.076 mg/ml, dissolved in mobile phase C without addition of tertiary amine.*

To optimize the separation time of the four tetracycline substances without loss of selectivity and peak symmetry, the influence of the acetonitrile concentration in the mobile phase on the capacity factor was determined. The results are demonstrated in Fig. 7. As expected, there is a drastic decrease of the capacity factor of III with a small increase in the acetonitrile concentration, but the capacity factors of I, II, and IV are influenced only to a limited extent. Figure 8 shows a separation of I, II, III, and IV by using a mobile phase consisting of 30% acetonitrile in pH 8.0 phosphate buffer and 0.0194 *M* *N,N*-dimethyloctylamine as the counterion.

Chromatography of Tetracycline Analogs and Potential Impurities—A suitable mobile phase composition to separate tetracycline analogs from their impurities was found to be pH 8.0 phosphate buffer with 30% acetonitrile and 0.0194 *M* *N,N*-dimethyloctylamine as the counterion (mobile phase A). The chromatographic data presented in Table III indicate that some of the substances have rather high capacity factors in mobile phase A, giving high retention times. Three alternative mobile phases were used, allowing regulation of the capacity factors over a wide range. Capacity factors for some of the tetracyclines in mobile phases B–D are also presented in Table III.

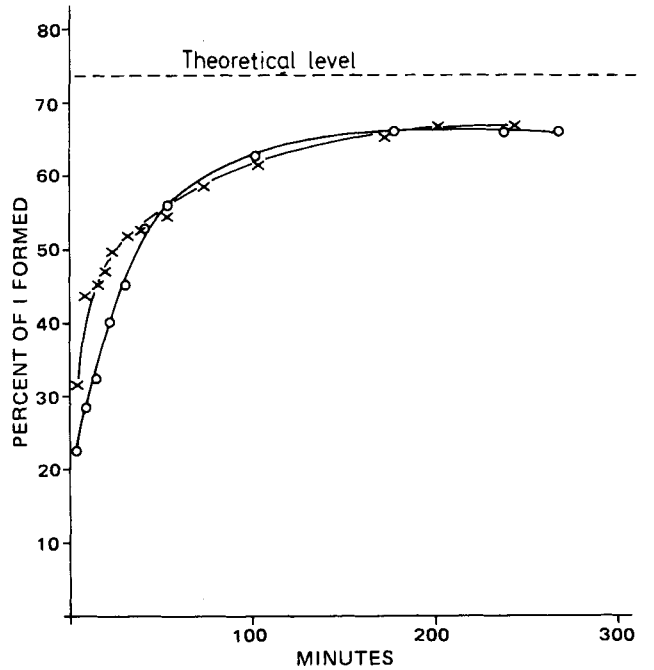

Figure 10—*Degradation rate of XVI to I. Key: ×, XVI (0.994 mg/ml) dissolved in pH 2.2 phosphate buffer; and ○, XVI (1.005 mg/ml) dissolved in pH 7.5 phosphate buffer.*

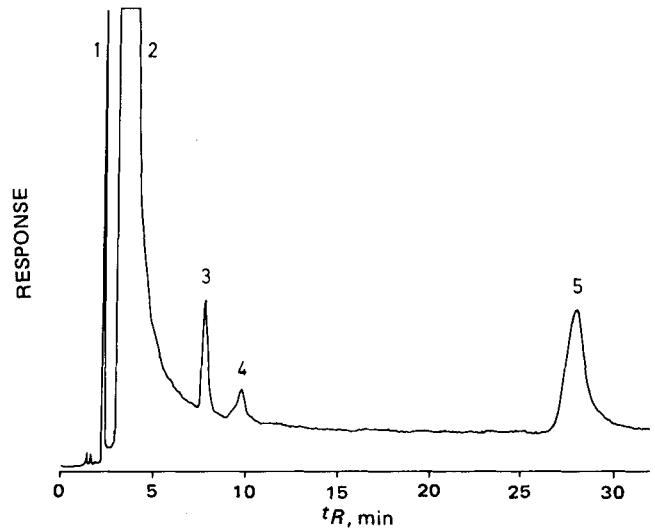

Figure 11—*Separation of I and its impurities from a tablet extract. Conditions were the same as in Fig. 8. Injection of 20 μl of a tablet extract with an initial concentration of 1.231 mg/ml is shown. Key: 1, II; 2, I; 3, IV (0.15%); 4, unknown impurity or tablet constituent; and 5, III.*

Stability of Tetracyclines—Tetracyclines have been reported (16) to undergo some degradation reactions like dehydration (at low pH) and epimerization between pH 3 and 5 in water solution. Stability tests of the tetracyclines were performed to exclude the possibility that the detected impurities were artifacts due to degradation during the chromatographic run, and to learn how to handle the dissolved substances before injection on the column. The commercial tetracyclines containing potential impurities were dissolved in the mobile phase without addition of the tertiary amine. A concentration of ~1 mg/ml of the tetracyclines was used, which allowed detection of the impurities to <0.1%. Repeated injections of the solutions were made over a minimum of 70 min, and the quantity of impurity was measured.

Two of the studied tetracycline substances showed significant degradation during the experimental period. Figure 9 shows the rate of rearrangement of XI to XIII. This study was performed by using a solution of XI with an initial concentration of 1.076 mg/ml. The substance was dissolved in mobile phase C without addition of the tertiary amine.

Figure 10 shows the degradation rate of XVI to I measured as percent I produced at pH 2.2 and 7.5 (phosphate buffer). The initial concentrations of (XVI) were 0.994 and 1.005 mg/ml, respectively. Mobile phase A was used for the quantitation of I produced. The molecular weights of I and XVI are 444 and 602.6, respectively, which means that 73.7% I produced corresponds to fully degraded XVI. The discrepancy between the theoretical and the observed I levels is not yet clearly understood. Compound XVI is a condensation product of I, lysine, and formaldehyde.

The described experiments show a fast degradation rate of XVI at acidic and neutral pH. This degradation means that there is a potential risk of formaldehyde liberation during therapy with XVI preparations. The liberation of formaldehyde during the degradation of XVI was determined by preparing the 2,4-dinitrophenylhydrazone derivative of formaldehyde followed by quantitation on a normal-phase column. Due to the fast degradation of XI and XVI, use of the described method is not recommended in the purity control of the drug substances.

Purity Control of Tetracycline Substances and Pharmaceutical Preparations—Figure 11 demonstrates the separation of tetracycline tablet extract containing small amounts of the impurities II, IV (0.15%), and III (0.6%). Table IV summarizes the content of impurities found in tetracycline, tetracycline analogs, and pharmaceutical preparations. There is good correlation of the impurity levels found in the drug substances and in the pharmaceutical preparations.

The precision of the method was evaluated to some extent by analyzing six samples containing 1 μg of III/ml. The relative SD was 6.5% at this concentration level. A concentration of 1 μg/ml corresponds to 0.1% impurity by injection of a tetracycline solution containing 1 mg/ml, which was the usual concentration of tetracycline used in the purity control studies.

Standard curves constructed by plotting peak areas *versus* sample concentrations show good linearity in the concentration range for all substances, with correlation coefficients >0.9994 in all cases.

REFERENCES

(1) A. A. Fernandez, V. T. Noceda, and E. S. Carrera, *J. Pharm. Sci.*, **58,** 443 (1969).

(2) P. P. Ascione and G. P. Chrekian, *ibid.*, **59,** 1480 (1970).

(3) E. Addison and R. G. Clark, *J. Pharm. Pharmacol.*, **15,** 268 (1963).

(4) J. P. Sharma, G. D. Koritz, E. G. Perkins, and R. F. Bevill, *J. Pharm. Sci.*, **66,** 1319 (1977).

(5) A. G. Butterfield, D. W. Hughes, W. L. Wilson, and N. J. Pound, *ibid.*, **64,** 316 (1975).

(6) J. H. Knox and J. Jurand, *J. Chromatogr.*, **110,** 103 (1975).

(7) *Ibid.*, **186,** 763 (1979).

(8) J. H. Knox and A. Pryde, *J. Chromatogr.*, **112,** 171 (1975).

(9) A. P. De Leenheer and H. I. C. F. Nelis, *ibid.*, **140,** 293 (1977).

(10) T. D. Sokoloski, L. A. Mitscher, P. H. Yuen, J. V. Juvarkar, and B. Hoener, *J. Pharm. Sci.*, **66,** 1159 (1977).

(11) B. Hoener, T. D. Sokoloski, L. A. Mitscher, and L. Malspeis, *ibid.*, **63,** 1901 (1974).

(12) K. D. Schlecht and C. W. Frank, *ibid.*, **62,** 258 (1973).

(13) R. E. Majors, *Anal. Chem.*, **44,** 1722 (1972).

(14) C. R. Stephens, K. Murai, K. J. Brunings, and R. B. Woodward, *J. Am. Chem. Soc.*, **78,** 4155 (1956).

(15) J. Hermansson, *J. Chromatogr.*, **152,** 437 (1978).

(16) K.-G. Wahlund, *ibid.*, **115,** 411 (1975).

(17) A. Sokolowski and K-G. Wahlund, *ibid.*, **189,** 299 (1980).

(18) D. Westerlund and E. Erixon, *ibid.*, **185,** 593 (1979).

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Hydration and Percutaneous Absorption III: Influences of Stripping and Scalding on Hydration Alteration of the Permeability of Hairless Mouse Skin to Water and *n*-Alkanols

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Abstract □ The influence of hydration on the permeability of stripped and scalded skins of hairless mice was investigated *in vitro* using water and *n*-alkanols as test permeants. Irrespective of pretreatment, the permeation rates of water, methanol, and ethanol were unaffected by aqueous immersion of skin sections in a diffusion cell, consistent with earlier data on unprocessed skins. The permeation rates of butanol and hexanol also were insensitive to hydration, differing from earlier studies on normal, intact skin in which both solutes' rates doubled after 10 hr of soaking. Following both pretreatments, the permeability of octanol declined over the first 5–10 hr of maceration, but remained invariant thereafter. The decline was most pronounced for the scalded skins. With untreated skin, octanol permeability initially increased and then declined before assuming a constant value. This study indicates that the barrier properties of the epidermis and dermis are not particularly sensitive to extended hydration except in the case of octanol. Scalding at 60° for 60 sec rapidly hydrates the skin, altering tissue permeability to about the same extent as a 10-hr (or longer) immersion in water at 37°. Octanol's unique hydration profile is explained by locating the origin of permeability decline in tissue beneath the horny exterior of the skin.

Keyphrases □ Permeability—of hairless mouse skin to water and *n*-alkanols after stripping and scalding □ Absorption, percutaneous—influence of stripping and scalding on permeability of water and *n*-alkanols, hairless mouse skin □ Hydration—alteration of permeability of stripped and scalded mouse skin to water and *n*-alkanols

It is known that the permeability of intact hairless mouse skin is altered by aqueous maceration, and that increases in permeation rates upon extended immersion of the skin in saline are a function of chemical structure for small, nonelectrolyte penetrants. Thus, the processes of hydration are complex, and probably involve more than one isolated phase of the skin membrane. A more thorough understanding of such hydration phenomena will add to the mechanistic understanding of the skin's barrier behavior.

Recently, effects of hydration on hairless mouse skin permeability were examined using water and *n*-alkanols as test permeants (1). These *in vitro* studies showed that hydration-induced permeability increases were a function of the penetrant lipophilicity. The permeabilities of the polar solutes, water, methanol, and ethanol, were not changed by hydration, while the permeabilities of the moderately lipophilic compounds, butanol and hexanol, asymptotically doubled in 10 hr of hydration. Permeation rates of the more lipophilic heptanol also increased to an asymptote, but only by ~50% in 10 hr. Octanol, the most lipophilic solute, showed an initial increase of ~50% in 5 hr, but then declined by ~25% by the 10th hr, undergoing a net increase in permeability of ~25%.

The skin hydration studies were extended to the Swiss mouse using water, methanol, ethanol, and butanol as permeants (2). In contrast to the hairless mouse skin results, water permeability increased up to 30 hr of hydration, and showed signs of leveling off between 30 and 43 hr. The permeabilities of methanol and ethanol also increased, but plateaued by 15 hr. Permeation rates of butanol increased over the first 15 hr and then declined almost linearly up to 48 hr. The hydration effect profile differences between the Swiss mouse and its hairless counterpart are