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In vitro and *in vivo* Study of the Biological Effects of Tri-Tetracyclines (New Tetracycline Complexes)

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Abstract. In vitro and *in vivo* experiments were performed with tri-tetracyclines prepared by mere dissolution of the tetracyclines in an aqueous solution of a recommended complexing agent, or using a preconstituted ready-for-use injection. Values of tri-tetracyclines of low minimal inhibitory concentration (MIC) were recorded. High antibiotic levels were found in the sera and in the tissues, especially in the lung after administration of tri-methacycline and in the kidneys after all tri-tetracyclines examined – indicating excretion via the kidneys. These facts correspond to enhanced water solubility without marked loss of lipid solubility.

Key Words
Tri-tetracyclines
New tetracycline complexes
MIC, concentration serum, concentration tissue
Lipid solubility, animal experiments

As a consequence of their favorable lipid solubilities, tissue affinities and high antibiotic activities, especially two tetracycline compounds have come to be used therapeutically in recent years: methacycline (6-methylene-5-hydroxytetracycline; MOTC) and doxycycline (6-α-deoxy-5-hydroxytetracycline; DOOTC), both of which can be regarded as derivatives of 5-hydroxytetracycline (OTC). OTC is frequently administered parenterally after conversion to a water-soluble derivative, whereas MOTC and DOOTC are generally given orally only. Although it is possible to bring about a high and prolonged serum antibiotic level with MOTC and DOOTC in small, single, oral doses, in emergency, in the case of gastrointestinal operations, or in order to attain rapidly an appropriate high serum level, their parenteral administration should be required.

An account is given below of observations made with tetracycline complexes prepared by ourselves. These complexes were prepared by dis-

solving the tetracyclines in an aqueous solution of the complexing agent immediately before administration, or were stored in a ready-for-use solution stable for longer periods. The concentration of the antibiotic in the solutions was 5%, and their pH values were 7.4–8.6. These tetracycline complexes are termed tri-tetracyclines since the tetracyclines form complexes with 3 molecules of tri-hydroxymethyl amino methan[1]. Data regarding the nature of the complexes will be reported elsewhere.

Materials and Methods

Antibiotics applied: 50 mg/ml tri-OTC (preconstituted, ready-for-use injection); 50 mg/ml tri-MOTC: MOTC. HCl was dissolved in an aqueous solution of the complex former immediately prior to administration; 50 mg/ml tri-DOOTC (preconstituted ready-for-use injection). The concentrations refer to the corresponding tetracycline hydrochlorides.

In vitro *examinations of antibiotic activity*. A quantitative comparison was made of the bacteriostatic activities of OTC.HCl, chlortetracycline (Cl-T), DOOTC.HCl, MOTC.HCl and the corresponding tri-tetracycline complexes, using the test strain *Staphylococcus var. aureus* 8537 NCTO 425. The test was a 2-fold, serial dilution of the antibiotic in broth. The inoculum consisted of 0.2 ml of a 1×10^{-3} dilution of a culture incubated overnight at 37 °C. Total final volume in each tube was 4 ml. The minimal inhibitory concentration (MIC) was recorded as the lowest concentration of the antibiotic-inhibiting growth of the microorganism, i.e., the last tube with clear broth.

In vivo *examinations of antibiotic activity*. Solutions of the tri-tetracyclines in the above concentration were injected into the marginal ear veins of 2- to 3-kg male rabbits and a study was made of the antibiotic concentrations of the serum as a function of time, and of the tetracycline contents of various organs at the end of the 8-hour experiment. (Since no toxic effects were observed, the tetracyclines were administered within the shortest possible time.) Blood samples taken in suitable intervals were centrifuged immediately after coagulation, and the antibiotic concentration of the serum was examined on the same day. Assays of sera were performed by an agar-well diffusion method with the use of large glass plates. Undiluted sera were used and *Staphylococcus aureus* 8537 NCTO 425 was the test organism. Levels were expressed in μg/ml based on comparison with the growth inhibition caused by known concentrations of the same drug (standards), after 20 h incubation at 37 °C.

After 8-hour blood sampling, some of the rabbits were killed by i.v. injection of air; 5-gram samples from the lung, the liver, the kidney and the gluteal muscles, and 1-gram samples from the spleen were measured, and after homogenization and triple extraction according to the method of SCHACH VON WITTENAU and DELAHUNT [1965], the antibiotic contents were determined by a method similar to that for the serum.

[1] D. P. 2 243 776

Table I. In vitro activity of tetracyclines and tri-tetracyclines versus *Staphylococcus aureous* 8537 NCTO 425

Tetracycline parent compound	Tetra-cycline	Tri-tetra-cycline	⊿MIC (tetra-cyclin tri-tetra-cyclin)	Standard error	Probability
Oxytetracycline	0.61	0.47	0.14	0.03	<0.01
Chlortetracycline (2 determinations)	0.60	0.50	0.10	–	–
Doxycycline	0.53	0.32	0.21	0.05	<0.01
Methacycline	0.44	0.23	0.21	0.04	<0.01

Average of MIC values in μg/ml from 8 separate tests (when not otherwise stated).

Examination of lipid solubility. The lipid solubilites of the tri-tetracyclines were determined by the method of SCHACH VON WITTENAU and YEARY [1963] in chloroform: 0.06 M phosphate buffer systems, changing the pH of the latter between 5.5 and 8.0. At least double determinations were performed from at least two parallel extracts. After extraction, the resulting pH of each aqueous phase was checked with a pH-meter (Radiometer, Copenhagen), and the chloroform-water distribution coefficients were plotted against the pH values. The distribution coefficients corresponding to pH 7.75 were extrapolated from the resulting curves.

Results

The MIC values obtained by comparison of the effectivities of the tetracyclines and the tri-tetracyclines *in vitro* are shown in table I. The *Staphylococcus var. aureus* 8537 NCTO 425 test demonstrated increased effects of all three tri-tetracyclines as compared to those of the corresponding parent-compound. The differences of MIC values of tri-tetracyclines and tetracyclines were in all cases highly significant. The study of other microorganisms and other tri-tetracyclines will be reported in a separate paper.

Following intravenous injection of either tri-MOTC prepared immediately prior to administration, or tri-OTC or tri-DOOTC stored in solution as the ready complex (preconstituted ready-for-use injection), the highest antibiotic concentration in the serum was measured after 1 h. The initial high tetracycline levels decreased to about half during the first 4 h, but for all preparations an antibiotic concentration exceeding the therapeutic

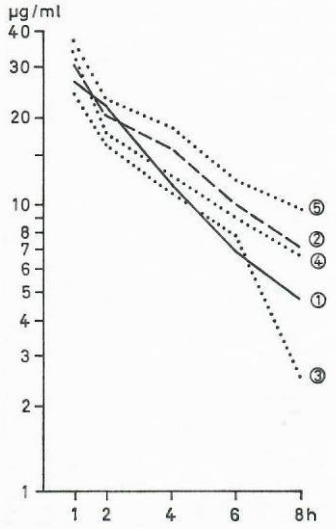

Fig. 1. 1 = Average serum values of 10 rabbits after i.v. administration of 20 mg/kg oxytetracycline (preconstituted injection); 2 = average serum values of 8 rabbits after i.v. administration of 15 mg/kg doxycycline (preconstituted injection); 3 = average serum values of 10 rabbit after i.v. administration of 10 mg/kg methacycline; 4 = average serum values of 6 rabbit after i.v. administration of 15 mg/kg methacycline; 5 = average serum values of 3 rabbit after i.v. administration of 20 mg/kg methacycline. All tetracyclines were administered in the form of tri-tetracyclines.

level in escess was observed at the last blood sampling. When three different doses of tri-MOTC were used, the serum values varied in direct proportion to the amount of the administered antibiotic, and paralleled each other (fig. 1.)

The antibiotic content of the tissues 8 h after the administration was in all cases several times higher than that of the serum, and substantially exceeded the therapeutic concentration (table II). The individual antibiotics follow each other in table II in the sequence of increasing *in vitro* activities. As regards the antibiotic contents of the organs, the highest antibiotic concentration was measured when tri-MOTC was administered. 10 and 15 mg/kg tri-MOCT resulted in roughly the same antibiotic levels in the organs, independently of the dose. The high antibiotic content of the lung after the administration of tri-MOTC was particularly striking. The antibiotic content of the liver and the muscles was about the same for all of the tri-tetracyclines. The tetracycline level of the kidneys was highest for

Table II. Drug concentrations (μg/g) in wet tissues and serum values of rabbits 8 h after i.v. injection of different tri-tetracyclines

	Dosis mg/kg	Number of animals	Serum μg/ml	Lung tissue	Lung sample / serum	Liver tissue	Liver sample / serum	Kidney tissue	Kidney sample / serum	Muscle tissue	Muscle sample / serum	Spleen tissue	Spleen sample / serum
Tri-OTC[1]	20	6	4.7	*17.5* $S\bar{x}$ 3.411 $p<0.001$	2.7	*27.37* $S\bar{x}$ 7.543 $p<0.001$	5.4	*187* $S\bar{x}$ 69.54 $p<0.05$	29.0	*26.8* $S\bar{x}$ 10.725 $p<0.02$	4.2	*16.53* $S\bar{x}$ 4.497 $p<0.01$	2.57
Tri-DOOTC[1]	15	6	7.0	*19.4* $S\bar{x}$ 1.967 $p<0.001$	2.8	*20.82* $S\bar{x}$ 1.834 $p<0.001$	3.0	*56.78* $S\bar{x}$ 11.376 $p<0.01$	8.1	*25.15* $S\bar{x}$ 3.952 $p<0.001$	3.6	*31.95* $S\bar{x}$ 4.311 $p<0.001$	4.6
Tri-MOTC	10	4	2.6	*32.1* $S\bar{x}$ 5.090 $p<0.001$	12.4	*26.37* $S\bar{x}$ 1.442 $p<0.001$	10.1	*99.45* $S\bar{x}$ 43.78 $p<0.05$	38.2	*21.15* $S\bar{x}$ 1.272 $p<0.001$	8.2	*40.33* $S\bar{x}$ 11.643 $p<0.05$	15.5
Tri-MOTC	15	6	6.6	*27.1* $S\bar{x}$ 8.295 $p<0.05$	4.1	*24.40* $S\bar{x}$ 3.69 $p<0.001$	3.7	*164.20* $S\bar{x}$ 38.11 $p<0.01$	25.0	*18.83* $S\bar{x}$ 4.01 $p<0.001$	2.8	*37.20* $S\bar{x}$ 2.88 $p<0.001$	5.6

$S\bar{x}$ = Standard error; p = probability.

[1] Preconstituted, ready-for-use injection.

Table III. Distribution coefficients ($K/CHCl_3/H_2O$) between chloroform and aqueous M 0.15 phosphate buffer of tetracyclines and tri-tetracyclines at pH 7.75 (average of 2–3 determinations)

Tetracycline parent compound	Tetracycline	Tri-tetracycline
Oxytetracycline	0.010	0.008
Demethylchlortetracycline	0.073	0.056
Methacycline	0.077	0.065
Doxycycline	0.390	0.320

all three antibiotics. The concentration of MOTC was the highest when the difference in dose of OTC and MOTC is taken into account and that of DOOTC the lowest. To characterize the tetracycline saturation of the organs, it is customary to use organ tetracycline content/serum tetracycline ratio. Lower values were found for tri-OTC and tri-DOOTC, and higher values for tri-MOTC, and the relatively high value for the latter, even in a small dose, is especially noteworthy. In the mathematical evaluation of the serum and organ values, the values of the regression coefficients were generally negative and, particularly as regards the kidneys, negative numbers of high absolute numerical value. After the administration of all of the tri-tetracyclines listed here, the 'constant term' (the tissue concentration corresponding to a tetracycline level just declining to zero concentration in the serum) was highest for the kidney.

The results of the chloroform-water distribution experiments are given in table III. The distribution coefficients for the individual tetracyclines were found to agree with values found in the literature [SCHACH VON WITTENAU and YEARY, 1963]. The chloroform-water distribution coefficients for the tri-tetracyclines were about 20% lower than those for the corresponding parent-compounds.

Discussion

In the *Staphylococcus aureus* test, significantly higher antibiotic activities were found for the tri-tetracyclines *in vitro*, than for those of the corresponding parent-compounds.

The high antibiotic activities were even more apparent in the *in vivo* experiments. It appeared from the examinations that the use of tri-OTC results in a high blood level in accordance with the literature data. The good biological effectiveness of the tri-tetracyclines emerges even more clearly in the cases of the highly lipid-soluble compounds tri-MOTC and

tri-DOOTC. It was found experimentally that a high serum antibiotic level is obtained when administering even a relatively low dose of tri-MOTC. 10 mg/kg tri-MOTC i.v. already caused saturation of the tissues with the antibiotic. This is shown by the same antibiotic contents of most tissues on the administration of two different doses at or above this threshold, and by the decrease of the tissue/serum quotient. Conversely, the antibiotic contents of the kidneys increase by a factor of more than 1.5, as an indication of excretion through the kidneys. Comparison with the literature data is possible only with certain reservations, since the reported earlier experiments were carried out mainly on dogs. The MOTC content of the dog serum measured by SCHACH VON WITTENAU and YEARY [1963] was higher; this was attributed by these authors to the extremely strong binding of the MOTC to the protein of the dog serum. The MOTC value of the rabbit serum examined in the present work is more comparable with the results of the studies of KUNIN [1962], who administered a similar amount to humans, but slowly, in a dilute solution. Compared to these, advantages result not only from the more rapid administration of the much more concentrated solution, but also from the higher serum values. The same applies to the observations made on the administration of tri-MOTC to humans [KAHÁN and HAMMER, 1974]. These observations on humans ensure also excretion of unchanged methacycline via the kidneys.

The rabbit serum antibiotic values obtained after the administration of tri-DOOTC correspond to the values measured by SCHACH VON WITTENAU and TWOMEY [1971] in dog serum. The results of the studies by SCHOOG [1971] with DOOTC, published after the completion of our experiments, are lower because of the smaller doses and the administration to humans. The antibiotic values in the kidney after the administration of tri-DOOTC was relatively low compared to the other organs and the other antibiotics; this can be, in the case of DOOTC, by being not excreted via the kidneys [SCHACH VON WITTENAU *et al.*, 1972].

The large absolute numerical values of the negative regression coefficients obtained on mathematical evaluation indicate the higher tissue tetracycline levels relating to the lower serum tetracycline levels. The constant term figuring in the regression constant calculated for the kidneys was the highest; this can be explained by the higher degree of lipid-solubility associated with the greatest pH gradient between the urine and the tubule membrane. The high absolute numerical values of the correlation coefficients of the kidney when using tri-DOOTC and 10 mg/kg tri-

MOTC were also conspicuous. Accordingly, the points lay close to the regression straight line for those compounds, which are lipid-soluble.

The protractedly high blood levels of the three tri-tetracyclines and the high antibiotic contents in the tissues can be explained by the 'optimum lipophility' of the tetracycline complexes formed (the tri-tetracyclines), the general importance of this having been pointed out already by BLACKWOOD and ENGLISH [1970]. The distribution coefficients of each of the tri-tetracyclines between chloroform and water phases differ only minimally from those of the starting compounds. The increase of the polarity by about 20% is of advantage in the preparation and stability of the injection. Moreover, if in itself it does not entirely explain the more favorable nature of the antibiotic activity *in vivo*, it may contribute to enhance penetration into the tissues and activity.

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