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Metal Ion–Tetracyclines Interactions in Biological Fluids. Potentiometric Study of Calcium Complexes with Tetracycline, Oxytetracycline, Doxycycline and Minocycline and Simulation of their Distributions under Physiological Conditions

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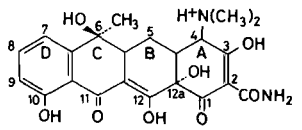
Equilibrium constants for the complexes formed between the calcium ion and a series of tetracyclines, i.e. *tetracycline, oxytetracycline, doxycycline, minocycline, were potentiometrically determined at 37 °C in aqueous medium NaCl 0.15 mol dm^{-3}. The distribution of the complexes was then simulated under physiological conditions at therapeutic levels of the drugs. Results are discussed concerning the possible effect of the calcium–tetracyclines interactions regarding the mode of action and the distribution of these antibiotics in the human body.*

Introduction

Tetracycline (I) is an amphoteric molecule which exhibits three proton-ionisation steps: the first is related to the proton dissociation of the C4 dimethylammonium ion, the second is associated with the C11–C12 diketone system and the third with the tricarbonyl methane system at the C1–C2–C3 positions [1, 2]



(I)

Due to its large number of potential binding sites, this molecule readily forms metal complexes in solution and it is not surprising that the mode of action of tetracycline and its derived antibiotics** in biological fluids is largely dependent upon the presence of certain metal ions.

Metal Ion–Tetracyclines Interactions in Hard Tissues

Tetracyclines display a strong affinity for and a deleterious impact on mineralizing systems such as teeth and bone. For example, the incorporation of these drugs into dental hard tissues during mineralization has resulted in such defects as enamel discoloration and hypoplasia in the tooth crowns of individuals who had been administered tetracycline from prenatal life through childhood [3]. Besides, depression of the growth rate of long bones of premature children receiving high doses of tetracyclines was observed [4]. A reduction of the foetal weight as a result of the administration of tetracycline to the mother has also been reported [4, 5].

The therapeutic and toxic dosages of any tetracycline have been stated as close to each other or even overlapping, the more so as the adverse effects have been proved cumulative [6].

From the various studies carried out on this topic [3, 7, 8], it can essentially be kept as a conclusion that tetracycline interferes with osteogenesis along two different pathways. Perrin [9] has shown that tetracycline can bind calcium atoms of bone apatite; the inhibition of calcification by tetracycline could therefore well be a direct blocking effect of the further growth of a mineral crystal. Previous *in vitro* observations have given support to this hypothesis at low tetracycline levels. In high concentrations of the drug, impairment of osteogenesis seems to be due

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**Oxytetracycline = 5-OH tetracycline, doxycycline = 6-deoxy-5β-hydroxy tetracycline, minocycline = 6-demethyl-6-deoxy-7-dimethylaminotetracycline.

to depressed collagen synthesis in addition to inhibited mineralization [7].

Both processes are metal ion dependent: *in vitro* studies have shown that tetracycline would deposit in mineralizing tissues only when the experimental conditions favoured the formation of a soluble calcium–tetracycline complex of ionic character in the liquid phase [10] – the fluorescence of the calcium–tetracycline complex has even been used successfully as a biological tag in developing tissues [11]. On the other hand, tetracycline affects collagen synthesis by complexing the Fe^{2+} ion which is a required cofactor in the hydroxylation of proline by protocollagen hydroxylase [3, 12].

Metal Ion–Tetracyclines Interactions in Antibacterial Action

Concerning the mode of transport of tetracycline through the membranes of bacteria, a tetracycline derivative, chlorotetracycline, was used by previous authors [13] as a fluorescent probe to investigate its own specific transport system in respiring *staphylococcus aureus* cells. Information about both transport and divalent metal ion roles was obtained on this occasion: it was proved that the antibiotics binds calcium or magnesium before it is transported to the apolar regions in the membrane.

Nevertheless, metal ions are generally considered as antagonists of the intrinsic antibacterial action of tetracyclines. For example, ferric, manganous and magnesium ions have been proved to reduce the inhibitory action of tetracycline against *pseudomonas aeruginosa* [14]. It has also been demonstrated that calcium and magnesium do limit the accumulation of tetracycline in *bacillus cereus* cells [15] and that magnesium does reduce the ability of tetracycline to bind to isolated t-RNA [16]. Conversely, a positive role for metal ions in the action of the drug was indicated by the ability of a zinc–tetracycline complex to bind DNA [17], but a more recent study did not confirm this observation [18]. Indeed, the study in question reports that tetracycline forms complexes with Cu^{2+}, Ni^{2+}, Co^{2+} that bind DNA when analysed in a filter-retention assay, but that other metal ions including Zn^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, Mg^{2+} and Ca^{2+} do not produce this effect. In particular, these results do not confirm a previous statement according to which, from the antimicrobial point of view, the tetracycline complexes with Ca^{2+} and Mg^{2+} would probably be the most important, because the concentrations of these cations and the large formation constants of their complexes with tetracycline dictate that tetracycline would exist *in vivo* as one of these two complexes [19].

All these studies essentially indicate that the metal ion cofactor should be considered in evaluating the mechanism of tetracycline action in biological systems, although its role in producing the antibacterial action of the drug has not yet been established.

Metal Ion–Tetracyclines Interactions in the Bioavailability of the Drug

Gastrointestinal absorption is the first process to be considered in defining the bioavailability of a drug [20]. As far as tetracyclines are concerned, it is well known that, among the different chemical species which can exert an influence upon their absorption, metal ions do play a very important role.

The best documented metal interactions on this process are undoubtedly those induced by iron. Indeed, numerous investigations were devoted to the impairment of tetracycline gastrointestinal absorption by iron sulphate and other salts in animals [21, 22] and man [23–25].

Some of the corresponding results clearly indicated the reciprocal inhibition of the absorption of simultaneously ingested $FeSO_4$ and tetracycline, which was attributed to the formation of complexes between Fe^{2+} and tetracycline [23]. It was also noted that the order of activity of different iron salts in this inhibition was the same as the order of the intestinal absorption of the involved iron compounds: the poorer the absorption of the iron salt, the weaker its action on the tetracycline absorption. But no interpretation was found to explain the weaker effect induced by Fe^{3+} comparatively to Fe^{2+}, whereas Fe^{3+} *in vitro* forms more stable complexes with tetracycline than does Fe^{2+} [24].

A certain number of pharmacological studies also dealt with the reduction of tetracycline intestinal absorption by zinc sulphate and other salts in rats [26] and in man [25, 27, 28]. Their most interesting result is that zinc sulphate reduced the intestinal absorption of tetracycline but had no effect on doxycycline absorption [25], whereas Fe^{2+} affected the absorption of doxycycline more than that of tetracycline [29].

The effects of calcium [20–22], magnesium [20, 30], even bismuth [27] were also investigated, leading to comparable conclusions.

Simulation of Metal Ion–Tetracyclines Interactions in Biofluids

All the studies mentioned in the above paragraph make clear the need for a better quantitative understanding of the equilibria between the various metal ions and the tetracyclines involved. For example, the simulated distribution of the metal complexes formed by a drug in a given biofluid is necessary to discriminate under which form it is likely to be expected.

Such simulations require (i) the experimental determination of the stability constants of all the complexes formed under the required conditions: some stability constants have already been calcu-

lated for complexes formed between metal ions and tetracyclines, but the related investigations are old [31] or the results incomplete [19, 32–34], new determinations are thus necessary, (ii) the use of appropriate computer models recent models have been developed for blood plasma metal complexes [35, 36], the latest and more sophisticated program [36], which can accommodate about 9000 species, has already been used by one of us to simulate metal interactions in a nutritive mixture for total parenteral nutrition [37] as well as in blood plasma [38, 39]

As Ca^{2+} is the most concentrated metal ion in various biofluids and as blood plasma is the most important among the latter, the present paper is devoted to the experimental study of the complex formation between Ca^{2+} and four tetracyclines tetracycline itself, oxytetracycline, doxycycline and minocycline, under plasma conditions The preliminary distribution of these antibiotics among the species they form with proton and Ca^{2+} is then computed on the basis of recent pharmacokinetic data obtained by one of us

Determination of the Formation Constants for Complexes of Calcium with Four Tetracyclines

Products

Tetracycline and oxytetracycline as free bases and doxycycline as hydrochloride were supplied by Pfizer lab , whereas minocycline hydrochloride was obtained from Lederle lab All these products were stored under an atmosphere of dried nitrogen Their content of free base and acid was potentiometrically controlled by Gran titrations [40], so they were then used without further purification Because the tetracycline antibiotics are unstable in aqueous media [13], fresh solutions were prepared daily prior to use

Hydrochloric acid and sodium hydroxide solutions for the titrations were prepared by diluting the contents of BDH Concentrated Volumetric Solution vials For alkali, these dilutions were made with deionised freshly boiled water under a nitrogen atmosphere, in order to prevent the carbonation of the solutions The titre of the latter was systematically controlled and standardised against potassium hydrogenophthalate Prolabo R P *pro analysi*, and from the features of the Gran titration plots were proved to be carbonate-free [40].

Sodium chloride *pro analysi* was supplied by Merck The stock solution of calcium was prepared from BDH Analar calcium chloride crystals This solution was made slightly acid by addition of hydrochloric acid, so as to prevent hydrolysis and absorption of carbon dioxide Its metal and proton contents were respectively checked by complexometric titrations using murexide as an indicator [41], and direct potentiometric readings

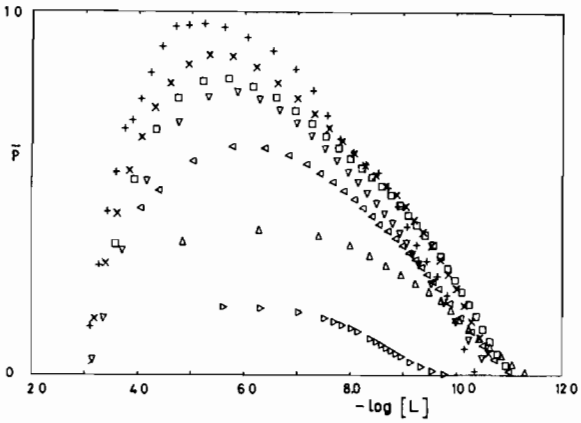

Fig 1 Experimental formation curve of the calcium–minocycline system The symbols are in the respective order of the metal to ligand ratios given in Table I for the system under consideration +, X, □, △, ▽, ◁, ▷

Potentiometric Equipment

The electrode arrangement was of the type

Glass electrode | Antibiotic, Ca^{2+}, NaCl (0 15 mol dm^{-3}) || NaCl | $HgCl_2$–Hg (saturated) (1)

using a Corning glass electrode and a saturated sodium chloride Ingold calomel electrode fitted in an Ingold Cell System

Potentiometric measurements were taken by means of a Beckman Model 4500 digital mV-meter, the reproducibility of which is 0 1 mV For each experiment, 20 cm^3 of initial solution were titrated against the standard sodium hydroxide solution containing NaCl 0 15 mol dm^{-3}, delivered from an ABU 12 Radiometer Autoburette

Experimental Conditions

Each initial solution for titration was prepared from known volumes of hydrochloric acid and calcium stock solutions, and from known volumes of the freshly prepared solution of the antibiotic under consideration It also contained NaCl 0 15 mol dm^{-3}, added as an ionic background to hold activity coefficients constant and to ensure isotonicity with blood plasma

The temperature was maintained at 37 00 ± 0 02 °C in the reaction cell by circulating thermostatted water All the titrations were performed under a constant bubbling of thermostatted, scrubbed, oxygen-free and carbon dioxide-free nitrogen The electrode system described above was calibrated in terms of hydrogen ion concentrations

TABLE I Antibiotic, Calcium and Mineral Acid Concentrations Used for the System under Investigation The NaOH titrant solution was standardised at 100 0 m*M* throughout this study

System	C_L (m*M*)	C_M (m*M*)	C_H (m*M*)
H^+–Tetracycline	9 24		10 00
	9 24		10 00
	9 24		19 96
	4 62		10 00
	9 24		20 00
Ca^{2+}–Tetracycline	9 24	4 875	20 52
	4 62	1 950	10 21
	9 24	0 975	20 10
	4 62	4 875	10 52
	9 24	2 925	20 31
	0 92	1 950	2 19
	0 92	1 950	2 19
H^+–Oxytetracycline	5 00		9 92
	5 00		9 92
	2 50		4 96
	2 50		4 96
Ca^{2+}–Oxytetracycline	5 00	0 975	10 02
	5 00	1 950	10 13
	5 00	4 875	10 44
	5 00	2 925	10 23
	2 50	0 975	5 06
	2 50	0 487	5 01
	1 00	1 950	2 19
	1 00	1 950	2 19
H^+–Doxycycline	10 00		19 84
	10 00		39 76
	5 00		9 92
	5 00		9 92
Ca^{2+}–Doxycycline	5 00	1 950	10 13
	10 00	0 975	19 94
	5 00	4 875	10 44
	10 00	2 925	20 15
	10 00	1 950	20 05
	5 00	0 975	10 02
	1 00	1 950	2 19
	1 00	1 950	2 19
H^+–Minocycline	43 50		84 39
	8 70		16 88
	8 70		16 88
Ca^{2+}–Minocycline	8 70	0 975	16 98
	8 70	1 950	17 09
	8 70	2 925	17 19
	4 35	4 875	8 96
	4 35	1 950	8 65
	4 35	2 925	8 75
	0 87	1 950	1 90
	0 87	1 950	1 90

On account of the low solubility of the tetracyclines and of their metal complexes, a slight opacity appeared in many solutions, sometimes from the very start of the complexation Consequently, the titrations were stopped when precipitation occurred in the solution, as indicated by a steady drift in the mV-meter readings

Table I summarizes the concentrations of the reactants used for the various experiments of the present study

Calculation of the Formation Constants

The general combined approach already used by one of us [38, 39, 42, 43] was employed throughout this study It essentially consisted of the two following stages

– first, the approximate values of the stability constants estimated from the formation curve of each system under consideration were refined by the MINIQUAD program [44], taking into account in turn every combination of the possibly existing species

– then, the sets of constants which were found the most likely to account for the experiments involved on the grounds of the usual numerical criteria (sum of squares, R factor), were graphically tested by comparing the experimental curves with the simulated ones, calculated by the PSEUDOPLOT program [45]

The protonation curves were based on the average number of protons bound to each ligand, according to the equation

$$\bar{r} = \frac{C_H + 2C_L - C_{OH} + [OH^-] + [H^+]}{C_L} \qquad (2)$$

where C_H and C_L stand for the total concentrations of the strong acid and of the antibiotic respectively, and C_{OH} the total concentration of hydroxide added at a given experimental point

The formation curves of the calcium–antibiotic systems were based on the average number of ligands bound to each calcium ion, as obtained from the equation

$$\bar{p} = \frac{C_L - ([L] + [HL] + [H_2L] + \quad)}{C_M} \qquad (3)$$

where C_M represents the total concentration of calcium

More details on the whole general numerical technique involving the use of the MINIQUAD and PSEUDOPLOT programs have been given in previous studies [38, 39, 42, 43], so we will not describe it further in the present paper Nevertheless, it is of interest to mention the particular strategy which was developed here for the computation of the formation constants of each system Actually, these constants

TABLE II Stability Constants of the Complexes Formed by Calcium with Tetracycline, Oxytetracycline, Doxycline and Minocycline at 37 °C in Aqueous Medium NaCl 0 15 mol dm^{-3}. The general formula of a complex is $M_qL_pH_r$, S represents the MINIQUAD sum of squares of residuals, n is the total number of experimental observations used for these calculations

System	p	q	r	log β	±	S	n
H^+–Tetracycline	1	0	1	9 052	0 004	0 135 E – 05	197
	1	0	2	16 323	0 007		
	1	0	3	19 485	0 011		
Ca^{2+}–Tetracycline	2	1	2	25 540	0 028	0 238 E – 05	172
	2	1	1	17 618	0 041		
	2	1	0	8 731	0 052		
	1	2	0	8 671	0 075		
H^+–Oxytetracycline	1	0	1	8 665	0 004	0 704 E – 07	95
	1	0	2	15 775	0 006		
	1	0	3	18 996	0 009		
Ca^{2+}–Oxytetracycline	2	1	2	24 625	0 029	0 101 E – 05	129
	2	1	1	16 654	0 089		
	1	1	0	4 462	0 138		
	2	1	0	8 385	0 064		
	1	2	0	(7 884)	(0 036)		
H^+–Dosycycline	1	0	1	8 676	0 005	0 690 E – 06	128
	1	0	2	16 090	0 007		
	1	0	3	19 186	0 013		
Ca^{2+}–Doxycycline	1	1	1	13 058	0 040	0 136 E – 05	131
	2	1	2	25 263	0 049		
	1	1	0	5 600	0 058		
	1	2	0	8 885	0 132		
H^+–Minocycline	1	0	1	8 968	0 003	0 187 E – 05	211
	1	0	2	16 392	0 004		
	1	0	3	21 268	0 006		
	1	0	4	24 311	0 007		
Ca^{2+}–Minocycline	1	1	1	14 183	0 018	0 180 E – 05	211
	2	1	2	27 228	0 036		
	1	1	0	5 981	0 125		

were calculated in two steps, depending on the metal to ligand concentration ratios investigated The first range, devoted to the search of the mononuclear species, dealt with the metal to ligand ratios inferior or equal to 1, the second dealt with the 2 1 ratio, specific to the formation of possible binuclear species The concentrations corresponding to these various ratios are given in Table I

The exact computational approach was as follows We first refined separately the constants pertaining to each ratio range, then we introduced all the species found in the same model and refined them together When this overall refinement resulted in a better standard deviation for the complexes previously recognized as the main species in the separate ranges, its outcome was taken as the 'best' result Conversely, when the standard deviation of the constant of a binuclear species became worse or, even, when the constant itself was made negative during MINIQUAD refinement, the constant unequivocally determined (if any) in the 2 1 metal to ligand ratio was held constant during the overall refinement

Results and Discussion

Details on the refinements of the stability constants pertaining to each metal to ligand ratio range will be available elsewhere [46] Thus, only the final results are given in Table II

The formation constant of the M_2L complex for the calcium–oxytetracycline system was made nega-

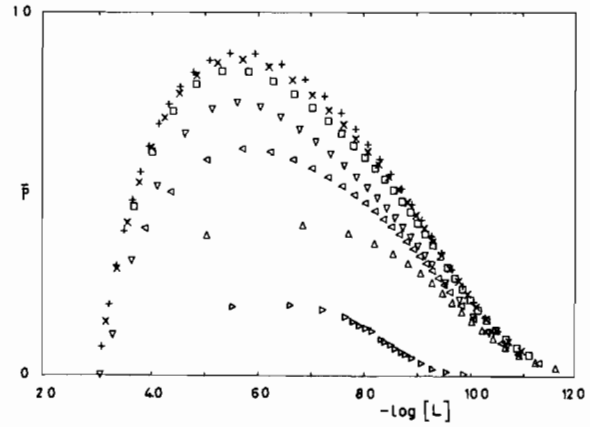

Fig 2 Simulated formation curve of the calcium–minocycline system, reflecting results in Table II The symbols are the same as in Fig 1

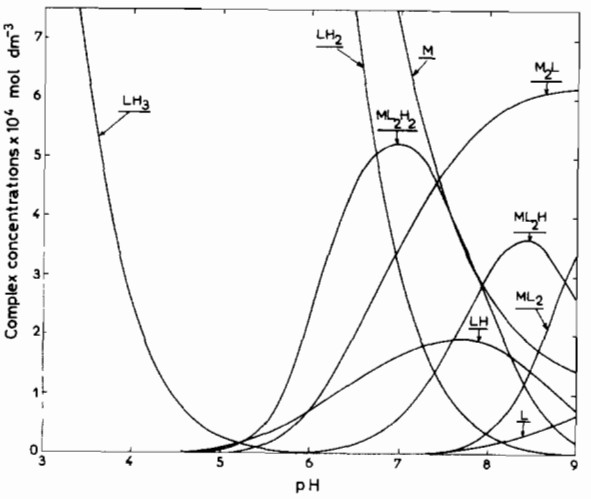

Fig 3 Ca^{2+}–Tetracycline system COMICS distribution of the complexes ($C_M = C_L = 2$ m*M*)

tive during the overall MINIQUAD refinement, so, as noted above, the value obtained in the specific 2 1 ratio was held constant in the final refinement It is also noteworthy that no binuclear complex at all was found for the calcium–minocycline system

As an example of our graphical comparisons, Fig 1 represents the experimental formation curve of the calcium–minocycline system It can be compared with the simulated one shown in Fig 2, which was obtained on the basis of the final results in Table II

In order to facilitate the comparison of the complex formation from one system to another, we have also calculated the respective distributions of all the species involved at different pH* values, using identi-

*pH is given as $-\log [H^+]$ in the present case

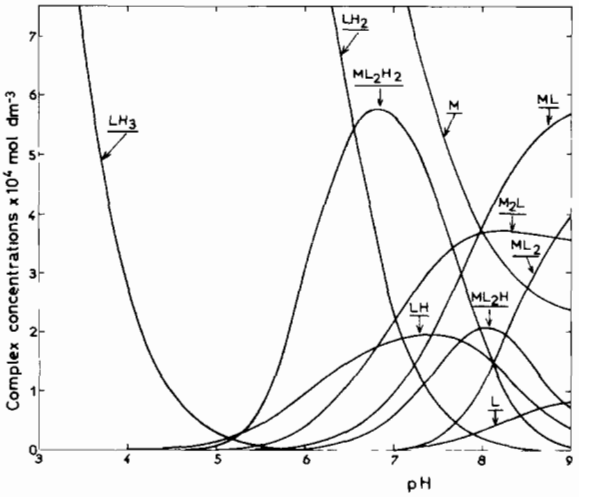

Fig 4 Ca^{2+}–Oxytetracycline system COMICS distribution of the complexes ($C_M = C_L = 2$ m*M*)

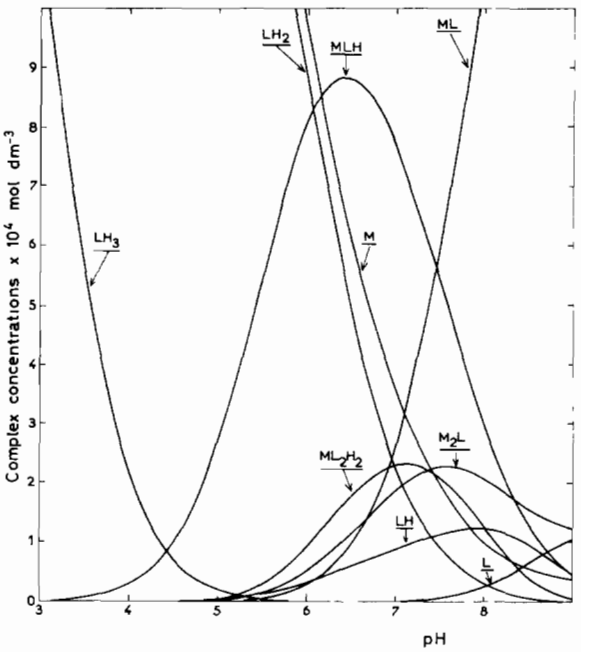

Fig 5 Ca^{2+}–Doxycycline system COMICS distribution of the complexes ($C_M = C_L = 2$ m*M*)

cal reactant concentrations Moreover, since M_2L as well as ML_2 complexes were formed, we have considered equal total concentrations for the calcium ions and the antibiotics (2×10^{-3} mol dm^{-3}) The related curves are drawn in Figs 3 to 6

A careful examination of these curves makes it clear that a certain similarity exists between the calcium complex formation with tetracycline and oxytetracycline on the one hand, and with doxycycline and minocycline on the other hand Indeed,

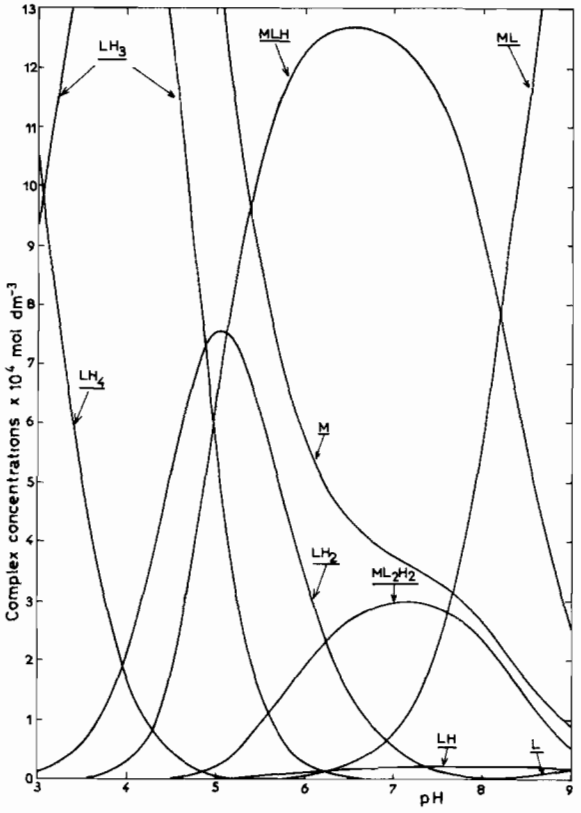

Fig 6 Ca^{2+}–Minocycline system COMICS distribution of the complexes ($C_M = C_L = 2$ m*M*)

the metal complexation is negligible up to pH 5 for the first two ligands, the concentration of ML_2H_2 is very important with regard to the other species as it reaches its maximum level at pH 7, then ML_2H, M_2L and ML_2 for tetracycline, ML_2H, ML, M_2L and ML_2 for oxytetracycline, become respectively significant For the two latter ligands however, the metal complexation is already noticeable from pH 4 and, although ML_2H_2 still reaches its maximum concentration at pH 7, it remains a minor species in comparison with MLH, which is the most predominant complex in the low pH range Moreover, in spite of the existence of a M_2L species in the calcium–doxycycline system, doxycycline and minocycline behave similarly in forming a very concentrated ML complex at pH > 7

Let us now compare our own observations with those mentioned by previous authors

Day and coll studied the calcium–tetracycline system in aqueous medium using fluorescence as well as circular dichroism techniques [32] They noted a maximum fluorescence at pH 7 5, which was attributed to the chelation of Ca^{2+} at the diketone system of the tetracycline molecule (C_{10}, C_{11}, C_{12}) The marked decrease in fluorescence at pH 9 and above was interpreted as a confirmation of the A-ring binding theory formerly put forward by Mitscher and coll [47, 48]. The involvement of the A-ring was proved to take place at the dimethylamino group, the calcium ion being bonded between the C_4 and C_{12a} sites It was also established that the binding of Ca^{2+} to the A-ring did not preclude the BCD-ring binding Yet, according to these authors, the complexes formed were in the 1 2 metal to ligand ratio, whatever the pH

We must remark at this stage that the characterisation of such a metal to ligand ratio in a solution which contained only 1×10^{-6} mol dm^{-3} of tetracycline together with 1×10^{-2} mol dm^{-3} of calcium would seem to be due to very high stability constants, whereas the authors calculated these as only 5 90 and 7.04 for pH 7 5 and 9.0 respectively

In addition, a former circular dichroism study by Newman and Frank [19], made at pH 7 4 in 90% methanol for different metal to ligand ratios varying from less than 1:1 to 2 1, showed the existence of a M_2L complex for the same system, and attributed the bond of the second calcium ion to the C_1–C_{12} site An older spectrophotometric study had also been made on the calcium–tetracycline system from pH 1 to pH 8 [20], but the authors did not supply any information on the complex stoechiometry and stated that only the diketone function was involved in the coordination, which has not been confirmed since then (see above).

Although the predominance of a 1 2 metal to ligand complex seems rather unlikely under the conditions of reactant total concentrations used by Day and coll [32], it is noteworthy that the maximum of fluorescence they observed at pH 7 5, roughly coincides with the maximum concentration of the ML_2H_2 species shown in Fig 3, which does denote the 1 2 metal to ligand ratio In the same manner, we can observe in Fig 3 that ML_2H reaches its maximum concentration near pH 9 and that its decreased importance above this pH corresponds to the increase of the ML_2 concentration This also tends to confirm the conclusions drawn by Day and coll [32] concerning the coordination sites

Indeed, it is obvious that the calcium ion can only be bound to the diketone system of tetracycline in ML_2H_2 [19], whereas the dimethylamino group is likely to take part in the coordination at the (C_4, C_{12a}) site for LH, as well as the diketone system at the (C_{10}, C_{11}) site for L, in the ML_2H species Nevertheless, the question remains to know how the M_2L species already found out by Newman and Frank [19], which appears the most significant in Fig 3, was not characterised by Day and coll under concentration conditions particularly favorable to its formation

As can be seen by comparing Fig 3 with Fig 4, oxytetracycline behaves towards calcium in a very similar way to that of tetracycline, except that the

TABLE III Concentrations of Tetracycline and Some of Its Derivatives in Blood Plasma at Various Times Following Administration (in mol dm^{-3}) For tetracycline, doxycycline and minocycline, the concentrations respectively correspond to a dose of 275 mg, 200 mg and 200 mg injected intravenously [50] For oxytetracycline, the concentration corresponds to an oral dose of 200 mg [51]

Times	Tetracycline	Oxytetracycline	Doxycycline	Minocycline
1 hr	$6\,93 \times 10^{-6}$	–	$1\,29 \times 10^{-5}$	$7\,21 \times 10^{-6}$
2 hr	–	$3\,04 \times 10^{-6}$	$1\,05 \times 10^{-5}$	–
4 hr	–	–	$8\,64 \times 10^{-6}$	$5\,77 \times 10^{-6}$
6 hr	$2\,34 \times 10^{-6}$	–	$7\,09 \times 10^{-6}$	–
8 hr	–	–	$5\,99 \times 10^{-6}$	$3\,85 \times 10^{-6}$
12 hr	$2\,21 \times 10^{-6}$	–	$4\,97 \times 10^{-6}$	$3\,15 \times 10^{-6}$
24 hr	$3\,60 \times 10^{-7}$	–	$2\,66 \times 10^{-6}$	$1\,57 \times 10^{-6}$

TABLE IV Simulated Distributions of the Tetracyclines into Their Complexes with Proton and Calcium under Blood Plasma Conditions at Various Times Following Administration The conditions are the same as in Table III The percentages inferior to 0 01% are omitted For all these distributions, the free calcium concentration largely exceeded 99 5% of the total one, fixed at $2\,45 \times 10^{-3}$ mol dm^{-3} pH was fixed at 7 40

Tetracycline after 1 hr		Tetracycline after 24 hr	
LH^-	1 57%	LH^-	1 55%
LH_2^0	1 16%	LH_2^0	1 15%
$ML_2H_2^0$	0 01%	$ML_2H_2^0$	–
M_2L^{++}	97 20%	M_2L^{++}	97 25%
L^{--}	0 03%	L^{--}	0 03%

Oxytetracycline after 2 hr	
LH^-	3 30%
LH_2^0	1 69%
$ML_2H_2^0$	0 02%
ML^0	12 71%
M_2L^{++}	82 07%
L^{--}	0 18%

Doxycycline after 1 hr		Doxycycline after 24 hr	
LH^-	0 28%	LH^-	0 28%
LH_2^0	0 29%	LH_2^0	0 29%
MLH^+	16 65%	MLH^+	16 57%
ML^0	14 56%	ML^0	14 50%
M_2L^{++}	68 19%	M_2L^{++}	68 33%
L^{--}	0 02%	L^{--}	0 01%

TABLE IV *(continued)*

Minocycline after 1 hr		Minocycline after 24 hr	
LH^-	0 21%	LH^-	0 19%
LH_2^0	0 23%	LH_2^0	0 20%
MLH^+	85 96%	MLH^+	85 56%
$ML_2H_2^0$	0 03%	–	
ML^0	13 56%	ML°	14 06%
L^{--}	0 01%	–	

formation of a ML complex has been evidenced for the former This result essentially confirms the conclusions of a previous investigation made by Ibsen and Urist [49], who postulated that ML and M_2L were the chief molecular species occurring *in vivo*, although higher metal to ligand complexes may exist The fact that they did not characterise ML_2H_2, which is the predominant species at pH 7 4 in Fig 4, clearly stems from the high metal to ligand ratios they investigated, for which the formation of this complex is highly unfavourable

Simulated Distributions of the Calcium–tetracyclines Complexes under Physiological Conditions

The stability constants determined above were used to simulate the distribution of the various calcium–tetracyclines complexes formed in human blood plasma following administration of these antibiotics

Choice of Simulation Data

The total concentration of calcium used for these calculations was taken as $2\,45 \times 10^{-3}$ mol dm^{-3} This value represents the total exchangeable concen-

tration of this metal in blood plasma, derived from the data of the latest computer simulation model of the metal–ion equilibria in this biofluid [36].

As for the concentrations of the four tetracyclines, the calculations carried out on tetracycline, doxycycline and minocycline made use of pharmacokinetic data recently determined by one of us [50, 51], which are briefly summarized in Table III For the sake of comparison, a value taken from literature concerning oxytetracycline [52] is also shown in Table III It is noteworthy that the plasma concentrations of tetracycline arise from an injected dose of 275 mg instead of 200 mg for doxycycline and minocycline, whereas the oxytetracycline one was derived from an oral dose of 200 mg For this reason, all these concentrations could not be compared with one another from a pharmacological point of view, but their similar orders of magnitude still provide a useful background to show the contrasting corresponding complex distributions

Simulation Program

The COMICS program [53] was used throughout these simulations

Results and Discussion

The simulated distributions of tetracylines into the complexes they form with proton and calcium did not notably vary within the time interval following drug administration which was investigated Accordingly, Table IV groups only the results corresponding to the limits of this interval, except the case of oxytetracycline, for which the only available data was related to the time of 2 hr after oral administration

The examination of these distributions suggests the following remarks

–whatever the period of time elapsed after drug administration, the various tetracyclines can be considered as nearly totally complexed by the calcium ions normally occurring in plasma Indeed, the calcium concentration used in the calculations does not take into account the interactions of the other low-molecular-weight ligands present in the medium, but it can by no means be inferior to the free concentration experimentally measured as 1 14 $\times 10^{-3}$ mol dm^{-3} [54] On the other hand, the total concentrations of the tetracyclines used encompass the fraction interacting with proteins [55] as well as that which combines with other metal ions, this means that calcium remains in any case in very large excess with respect to tetracyclines in normal blood plasma Consequently, the concentrations of free tetracyclines are always quite negligible with regard to the total ones This conclusion could be of some importance for the discussion of the ability of tetracyclines to diffuse from the blood stream to the environmental tissues, which was so far almost exclusively based on partition coefficient considerations affecting the free molecules [56]

– if we compare the distribution of the four tetracyclines in Table IV, we can notice that the protonated species are quite negligible for doxycycline and minocycline, whereas they represent about 3% of tetracycline and 5% of oxytetracycline This observation derives from the fact already mentioned in the above chapter that doxycycline and minocycline will complex calcium at lower pH than the other two antibiotics Apart from this, the striking difference between the four tetracyclines lies in the absence of M_2L for minocycline, which contrasts with the high percentage of this species in the other three cases As for the discrimination of the neutral species which can allow the drugs to diffuse into cell membranes, only 1% of tetracycline (as LH_2) fulfill this condition whereas about 15% of the other three antibiotics (essentially under the ML form) can do it These particularities do not correlate very closely with the *in vivo* observations made by English [57] on the tetracycline activity against *staphylococcus aureus* in mice, but this is not really surprising

(i) indeed, calcium is not the only metal likely to form complexes with tetracyclines in blood plasma First of all magnesium, which also denotes a concentration largely in excess with respect to those of the drugs [36], is known to give rise to stable complexes with tetracyclines [19, 20, 49]. So, the lack of correlation observed rather indicates that further studies devoted to the coordination of other metal ions are necessary before a more realistic distribution of the tetracyclines in blood plasma can be obtained

(ii) on the other hand, it is noteworthy that neutral species may diffuse more or less easily, according to their own structure In particular, it is obvious that their interactions with the solvent can influence both their ability to diffuse and their diffusion rate The same kind of observation could be made for charged species with regard to their elimination rate All this means that the study of the physicochemical properties of the main complexes present in the biofluid under consideration could help to enable one to account for clinical observations in a more complete way, or to state more precise predictions But the discrimination of these main complexes anyhow implies that the reliable distribution mentioned above can be simulated beforehand

To conclude, as the free concentrations of the tetracyclines have been proved quite negligible with regard to the complexed ones in the patients' blood plasma during treatment, the present study shows that the partition coefficient should not be the only parameter to be considered to predict the physiological activity of these antibiotics *in vivo* Furthermore, although this study has established that the role of calcium is very important towards tetra-

cyclines in blood plasma, it has also made it clear that the knowledge of the interactions of the drugs with other metal ions, first of all magnesium, is necessary before reliable predictions can be made about their mode of action in the human body This will constitute our next objective

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