Stability study of tetracyclines with respect to their use in slow release systems

V. C. HONNORAT-BENABBOU[1], A. A. LEBUGLE[2], B. SALLEK[3], D. DUFFAUT-LAGARRIGUE[4]
[1]*Laboratoire de Parondotologie, Faculté d'Odontologie, 3 chemin des Maraîchers, 31062 Toulouse cedex, France*
[2]*Laboratoire des Matériaux, Equipe sur les Phosphates, ENSCT-INPT, ESA CNRS 5071, 38 rue des 36 Ponts, 31400 Toulouse, France*
[3]*Laboratoire de Physico-Chimie des Matériaux, B.P. 133 Faculté des Sciences IBNTOFAIL, 14000 Kenitra, Morocco*
[4]*Laboratoire de Sciences Biologiques, Faculté d'Odontologie, 3 chemin des Maraîchers, 31062 Toulouse cedex, France*
E-mail: lebugle@cict.fr

In the aim of optimizing implantable slow-release systems for the local delivery of antibiotics, the stability of tetracyclines was studied in water at 37 °C or under γ irradiation. Four tetracyclines in their chlorhydrate form were chosen depending on their hydrophilic/hydrophobic balance. Their chemical stability was established by HPLC, and biological stability by bacteriological tests. It was shown that methacycline and doxycycline are stable in water for three days. Tetracycline and minocycline exhibit limited decomposition (less than 10%) under the same conditions. So, *in vitro* drug release for at most three days, appears to be possible. Besides, all four tetracyclines either in powdered form or included in a calcium phosphate matrix, kept their bacteriological activity after γ irradiation at 32.4 kGr. Consequently, the *in vivo* study of these implantable slow drug release systems, can be carried out.

1. Introduction

Tetracyclines have several therapeutic applications in dental surgery because of their broad antibacterial spectrum, their efficient diffusion in bone and their inhibitory effect on collagenases and bone resorption [1, 2]. For these reasons, they are used widely in the treatment of various clinical types of periodontitis [3–5]. However, long-term oral administration leads to side effects such as digestive disturbances, tooth discoloring and enamel dysplasia. In addition, some risks of nephrotoxicity increased with pre-existing kidney deficiency, have also been mentioned [1, 6, 7]. In order to minimize these effects, research has been devoted to the local and slow release of these antibiotics [8–13]. Consequently, it is possible to reach a very high local concentration of antibiotic while keeping systemic concentrations low [14]. Different carriers have been tested, such as collagen, hydroxyapatite, plaster of Paris and β tricalcium phosphate mixture [15–21]. However, with these carriers the antibiotic is often released too quickly.

In a previous study, slow release of aminosides [22] was obtained using a calcium phosphate matrix in which the antibiotic was included by compaction. In this system, release is controlled by adsorption/desorption on the carrier and by the implant porosity. Such a system could also be used for local slow release of tetracycline.

However, rational optimization of such implantable systems needs an *in vitro* kinetic study of drug release and an *in vivo* study of their biocompatibility and biodegradation. *In vitro* studies lead to long contact times between the tetracyclines and the dissolution medium, usually water. *In vivo* studies must be performed with sterilized implants, usually by γ irradiation. However, it has been reported that tetracyclines are unstable in solution and sensitive to light and irradiation [6, 23–26]. Before considering any application to slow drug release systems, the stability of tetracyclines must be established in the conditions required for *in vitro* and *in vivo* studies. This stability was examined from chemical and biological points of view. The results are reported here.

2. Materials and methods

The stability of four tetracyclines was studied: tetracycline, doxycyline, methacycline (Sigma) and minocycline (Wyth-Lederle). In order to obtain high solubility, the chlorhydrate forms were used. These tetracyclines are distinguishable by their Kp coefficients

TABLE I Amounts of unaltered tetracyclines at 37 °C in water, with time

	Tetra	Doxy	Meta	Mino
Initial	100%	100%	100%	100%
3° day	88%	97%	98%	91%
10° day	70%	94%	93%	79%

(liposolubility/hydrosolubility ratio) which are related to their bioavailability [6, 7].

The matrix (or carrier) was apatitic octacalcium phosphate named OCPa($Ca_8(PO_4)_{3.5}(HPO_4)_{2.5}(OH)_{0.5}$). It has the same Ca/P atomic ratio, 1.33, as triclinic octacalcium phosphate $Ca_8(PO_4)_4(HPO_4)_2 \cdot 5H_2O$, and presents the same apatitic crystalline structure as the mineral part of bones and teeth. Its synthesis and structure were established by Zahidi *et al*. [27] and by Lebugle *et al*. [28]. This apatite is very deficient, presenting numerous vacancies. It exibits outstanding physico-chemical properties, especially an excellent compaction ability [29], much higher than that of the carriers used in standard pharmacy. OCPa is biocompatible, rapidly biodegradable and osteoconductive. For these reasons, OCPa has already been used as a matrix for implantable slow drug release systems [22]. For the present study, OCPa was prepared using the synthesis method reported above. Chemical analysis showed its Ca/P atomic ratio to be 1.33. The Fourier transform infrared (FTIR) spectrum and X-ray diffraction (XRD) pattern correspond to those already reported for this compound.

The implantable systems were made from powdered mixtures of 2% (weight) of one of the tetracyclines.base, 5% (weight) of dextran (Sigma Clinical Grade), made up to 100% with OCPa. Dextran was added to increase the porosity. Implants having a weight of about 250 mg, were obtained by compaction of mixtures described above, with a Korsch Edko alternative machine, fitted with 6 mm flat punches. The implants exhibited a ''Stockes hardness'' of 8.5 DaN. They were used as is or after γ ray sterilization at 32.4 kGr (required minimal dose: 25 kGr [23]).

The chemical stability of tetracyclines was studied by checking for changes in High Performance Liquid Chromatography (HPLC) chromatograms (number of peaks, retention time, peak area) after maintaining the drugs in the experimental conditions needed for *in vitro* and *in vivo* studies. Analysis was performed with a Hyperchrome Ultrasept E.S. 100 R.P. 8 (length: 125 mm), a flow rate of 800 ml/min, an injection loop of 20 ml and detection at 280 nm. The mobile phase adapted from the European Pharmacopea requirement was composed of 25 volumes of an EDTA solution (4 g/l), 27 volumes of dimethylformamide and 50 volumes of an ammonium oxalate solution (25 g/l). The mobile phase was carefully adjusted to pH 7 with a tetrabutylammonium solution (104 g/l).

Stability in deionized water was studied with solutions at 100 ppm of tetracycline, maintained at 37 °C under stirring for various times. Experiments were performed in borosilicate brown glass bottles to avoid photodegradation. Samples were collected on days 1, 3 and 7, and stored frozen at − 20 °C to be further analyzed by HPLC. It was checked that all samples remained perfectly stable during storage at this temperature, as previously observed by Rose *et al*. [30].

Stability to γ irradiation was established by HPLC of irradiated or non-irradiated solutions containing 100 mg/l of antibiotics.

Moreover, because the energy due to irradiation is able to induce a solid state reaction between the tetracyclines and calcium phosphate matrix, some implants, before or after sterilization, were crushed and stirred in 50 ml of deionized water at room temperature for 30 min. After filtration, samples were analyzed by HPLC.

Bacteriologic stability was studied to check if tetracyclines included in the implants kept their bacteriostatic activity after γ sterilization. Tests were carried out either on solutions of tetracyclines extracted from pellets, or directly on implants themselves. Bioassays were performed with *Prevotella intermedia* (Pasteur strain) which are currently found in periodontitis [31–34] or with *Prevotella intermedia* strains collected from patients. Growth inhibition was observed on Columbia jelly dishes (Gelose Columbia 19.5 g, Cystéine HCl 0.08 g, Hemine 5 ml, Menadione 50 ml, sheep-blood 25 ml).

In the first set of experiments, a pellet containing one of each antibiotic, was crushed and stirred in 50 ml of sterilized water. One ml of this solution was added to 10 ml of Columbia jelly before pouring into the dishes (CMI of 10 mm/ml) [35]. Two series were performed. These dishes were inoculated with *Prevotella intermedia* for the first series, and with *Prevotella intermedia* strains collected from patients for the second series, and incubated 72 h at 37 °C in anaerobic conditions (Forma Scientific Bioblock). Growth inhibition was observed at 72 h and this was compared to control dishes with only jelly inoculated with the Prevotella Strains.

In the second set of experiments, sterilized implants themselves were layed on a Columbia jelly dish previously inoculated with *Prevotella intermedia* strains [36, 37]. A tablet of OCPa without antibiotic was tested too, as a blank.

3. Results and discussion

3.1. Chemical stability in solution

The chromatograms of tetracyclines solutions, maintained at 37 °C for 1, 3 or 10 days, show that new peaks develop with time. Fig. 1, for example, shows the case of minocycline. These new peaks are due to decomposition of the antibiotic and were quantified from peak areas. Amounts of unaltered antibiotics were determined in this way, and are reported in Table I. It can be seen that stability in water depends on the antibiotic. Thus, methacycline and doxycycline were pratically unaltered after three days, whereas limited decomposition occured with tetracycline and minocycline. At 10 days, decom-

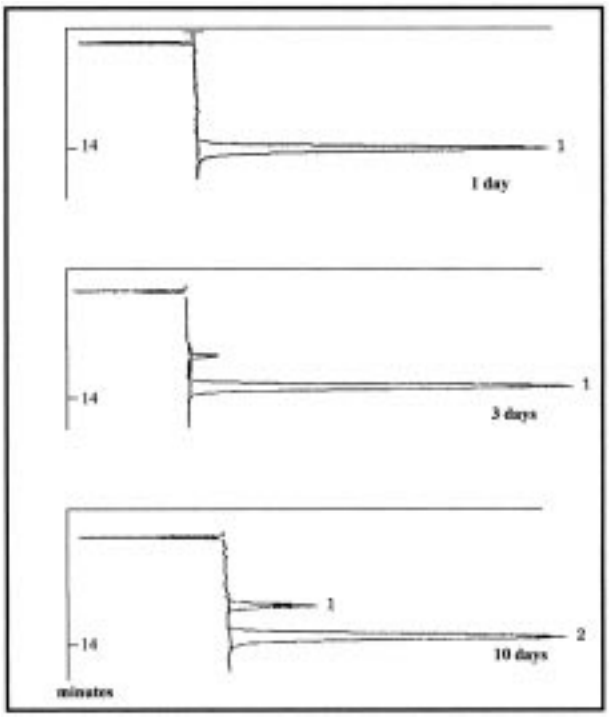

Figure 1 Chromatograms of minocycline solutions maintained at 37 °C for various times.

position continued, but doxycycline and methacycline appeared more stable than the other ones.

3.2. Chemical stability after γ irradiation

Chromatograms of solutions prepared with irradiated and unirradiated tetracyclines were strictly the same, as can be seen for example with minocycline (Fig. 2). There are no secondary peaks or changes in relative retention time. These observations indicate that none of the four tetracyclines studied were degraded by γ ray irradiation at 32.4 kGr during the sterilization process. These results seem to be in disagreement with those reported by Davies *et al*. [25]. It should be noted that these authors used radiation with a completely different wavelength. It is well known that the stability of chemical bonds is closely dependent on this parameter.

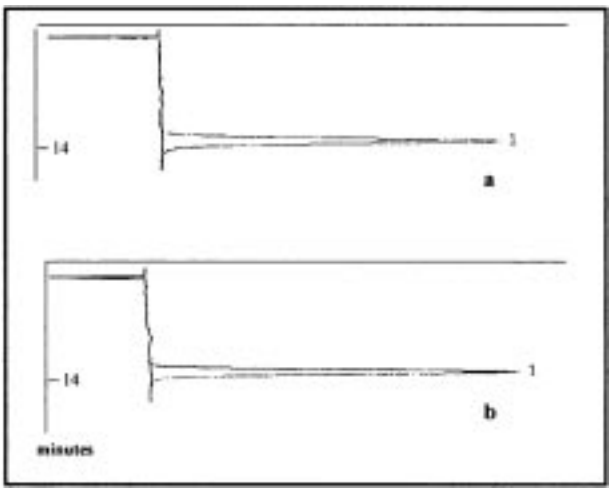

Figure 2 Chromatograms of minocycline solutions, (a) before sterilization; (b) after sterilization.

Chromatograms of solutions obtained by extraction of tetracyclines from implants, whether sterilized or not (after crushing and stirring in 50 ml of water), were identical. No additional peaks or modifications of relative retention time appear after sterilization (e.g. minocycline, Fig. 3). As a consequence, despite its high energy, γ irradiation does not induce any solid state reaction between calcium phosphate and tetracyclines.

3.3. Biological activity

Complete growth inhibition was observed with all tetracyclines extracted from sterilized implants. Such inhibition did not appear with control dishes (Fig. 4). This demonstrates that tetracyclines remained active after the sterilization process.

A growth inhibition zone appeared around implants containing antibiotics, whereas no inhibition zone was observed around the antibiotic-free implant (Fig. 5). This result demonstrates that tetracyclines can diffuse from the implant after γ irradiation sterilization and remain active. However, interaction with calcium ions is known to reduce tetracycline activity [38]. It should be pointed out that the solubility of apatitic calcium phosphate is very low, so the concentration of calcium ions will be low too. As a consequence, interaction in solution between tetracyclines and calcium ions will be weak, thus allowing the biological activity to be preserved.

4. Conclusions

The results of this study carried out on four tetracyclines showed that these antibiotics are relatively stable. Indeed, they can be sterilized by γ irradiation either as powders, or dispersed into an OCPa implant matrix, without loss of biological activity, or degradation of the molecules. In the same way, these antibiotics are practically stable in water at 37 °C up to three days. Thus, rational optimization of their use in implantable systems can be considered. Indeed, the *in vitro* study of their release kinetics from implants becomes possible and will allow their composition to be adjusted to suit the

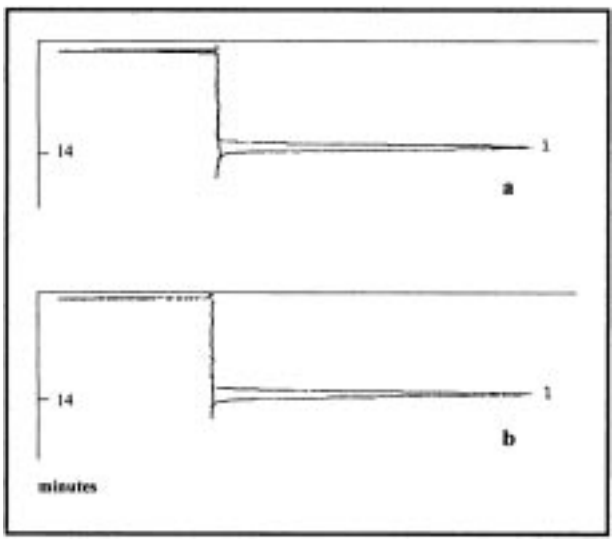

Figure 3 Chromatograms of solutions made with minocycline extracted from implants, (a) before sterilization; (b) after sterilization.

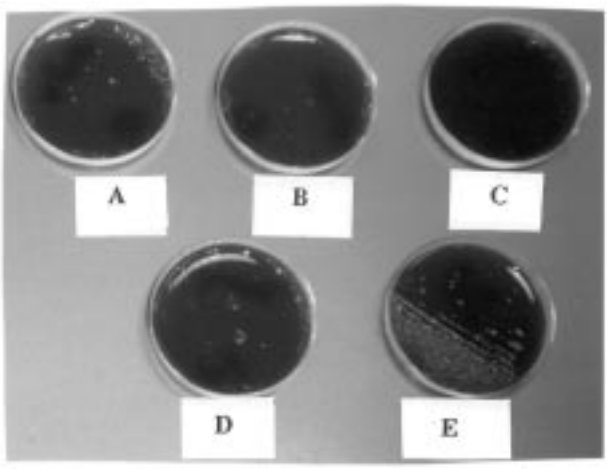

Figure 4 Biological activity of antibiotics extracted from sterilized pellets, on gelose after inoculation with *Prevotella intermedia* strains. (A: methacycline; B: minocycline; C: tetracycline; D: doxycycline; E: control dish.)

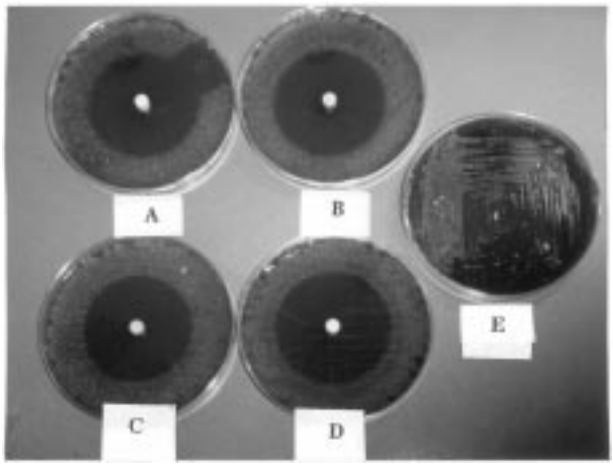

Figure 5 Biological activity of sterilized pellets containing antibiotics, on gelose previously inoculated with *Prevotella intermedia* strains. (A: tetracycline; B: methacycline; C: minocycline; D: doxycycline; E: control dish.)

applications. Due to stability with respect to γ irradiation, it will also be possible to examine their *in vivo* behavior, namely their biocompatibility and biodegradation, while investigating antibiotic diffusion both into the nearby tissues and systemically.

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