Antimicrobial properties of tetracycline and minocycline-montmorillonites

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ABSTRACT

The antimicrobial activity of adsorption complexes of antibiotics from the tetracycline family on sodium and calcium montmorillonite was evaluated for their use in human and veterinary medicine. Tetracycline (TC) and minocycline (MC) adsorption on Patagonian montmorillonite was studied as a function of pH using batch experiments, X-ray diffraction (XRD) and IR spectroscopy. The adsorption was high at low pH and decreased continuously as pH increased. Adsorption of MC was higher than adsorption of TC. TC and MC were intercalated into the interlayer space of montmorillonite as deduced from the increase of the basal spacing. IR spectroscopy suggested that NHR_3^+ and other polar groups were directly involved in the interaction with montmorillonite. The antimicrobial activity, measured by inhibition tests, was maintained by the antibiotics after adsorption on montmorillonite, whereas unreacted montmorillonite did not show any antibacterial activity. The excellent activity shown by the TC and MC complexes provides a promising field of application, in particular for the formulation of topical products.

1. Introduction

Properties such as colloidal particle size, crystalline structure, high specific surface area, charge and swelling capacity provide clay minerals optimum rheological behavior and excellent adsorption capacities to be used for therapeutic and cosmetic purposes (López-Galindo et al., 2007). Kaolinite, talc, some smectites (montmorillonite and saponite) and fibrous minerals (sepiolite and palygorskite) are found among the most important minerals for the human health care (López-Galindo and Viseras, 2004). Due to their high cation exchange capacity, smectites interact strongly with some drugs affecting their bioavailability (Aguzzi et al., 2007). This interaction can be advantageous in the design of modified-release systems that is one of the most interesting fields of pharmaceutical applications of clay minerals nowadays (Carretero, 2002; Aguzzi et al., 2007). Clay minerals fulfill chemical, physical and microbiological requirements that make them appropriate for use in pharmaceutical technology and dermopharmacy as excipients and as substances with adequate biological activity (López-Galindo et al., 2007). Some antibacterial inorganic materials were obtained using Ag^+ or Cu^+ exchanged montmorillonites (Hu and Xia, 2006). Clay minerals are present in solid (tablets, capsules and powder), liquid (suspensions and emulsions) and semi-solid (creams and pastes) administration forms for topical application, attributed to properties such as the capacity of adhesion to skin, or oral administration due to their chemical inertness and binding properties.

Samples of Patagonian montmorillonite were previously evaluated for their potential use in pharmaceutical formulations. The chemical and microbiological compositions make them safe materials for their use as pharmaceutical excipients (Viseras et al., 2006). Patagonian montmorillonite was calcined and ground and then subjected to cation exchange with silver ions. The silver modified montmorillonites showed good inhibition of the growth of *Eschericchia coli* whereas the natural, calcined and ground samples did not show any antibacterial activity (Magaña et al., 2008).

The use of antibiotics in human and veterinary medicine grew very fast since their discovery. Their use in preventive medicine and in the treatment of several infections turned these compounds into some of the most widely popular ones in the human and animal health care (Chambers, 2001). Tetracyclines include a group of antibacterial agents with common chemical structure and pharmaceutical actions, very frequently used against gram-positive and gram-negative microorganisms. These antibiotics were incorporated in animal food to favor healthy growth (Sarmah et al., 2006). The so-called tetracycline (TC) and minocycline (MC) are compounds from this antibiotic family. TC chemical properties were extensively studied (Stephens et al., 1956; Stezowski and Prewo, 1977; Lambs et al., 1983). TC and MC have different acidic groups and the possibility to adopt different ionic species and conformations depending on the pH of their aqueous solutions (Qiang and Adams, 2004). Electron donor groups present in TC and MC structures permit complex formation with cations such as Ca^{2+} and Mg^{2+} (Mackay and Canterbury, 2005; Gu et al., 2007).

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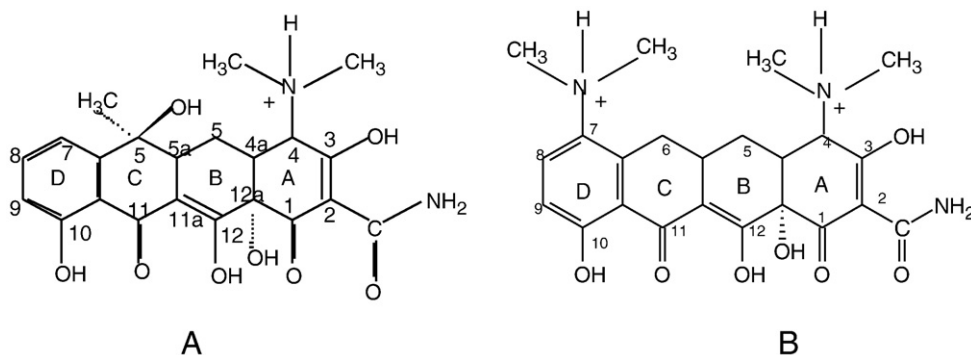
Fig. 1. Fully protonated tetracycline (A) and minocycline (B).

Tetracycline adsorption onto different clay minerals, including montmorillonite (Swy-2 and SAz-1) (Li et al., 2010), hectorite from California, kaolinite (hydrite-10) (Browne et al., 1980), palygorskite from Georgia (Chang et al., 2009c), and rectorite (Chang et al., 2009a), was previously studied. Browne et al. (1980) and Chang et al. (2009b) concluded that the interaction was related to the surface charge of the clay mineral particles. Aguzzi et al. (2005) studied the adsorption of several tetracyclines (tetracycline, chlortetracycline, methacycline, minocycline and doxicycline) on two pharmaceutical-grade smectites containing mainly montmorillonite. They concluded that the interaction between antibiotics and clay minerals was highly dependent on the contact time and mixing conditions used to disperse the clay mineral aggregates. The interactions between TC and Patagonian montmorillonite in aqueous dispersion were investigated in previous studies (Parolo et al., 2008b). The medium pH was a decisive factor in the TC affinity for the mineral because pH determines the ionic state and hence physicochemical properties of the antibiotic.

Montmorillonites and fluoromicas intercalated with organic compounds with antibacterial effects such as cetylpyridinium, cetyltrimethylammonium and norfloxacin were also studied (Dizman et al., 2007; Malachová et al., 2009). The organo-montmorillonites and fluoromicas showed excellent antibacterial activities. However, no studies were found on the antimicrobial activity of TC and MC adsorbed on montmorillonite.

The aim of this article is to compare the antibacterial activity of TC-montmorillonite and MC-montmorillonite. Batch adsorption studies at different pH were performed to select the optimum conditions for the formation of the antibiotic-mineral complexes.

2. Materials and methods

2.1. Materials

We used a representative sample of the bentonite found in the northern Patagonia, Argentina, belonging to Upper Cretaceous age. This sample was obtained from the bentonite deposit from Perito Moreno mine. The chemical analysis and physicochemical properties of these bentonites were previously reported by Lombardi et al. (2003), Vallés and Impiccini (1999) and by Viseras et al. (2006). The exchangeable cations are mainly Na$^+$. The chemical and microbiological compositions correspond to materials that are suitable for inclusion in pharmaceuticals formulations. The sample had a cation exchange capacity (CEC) of 1.04 meq/g as measured by the ammonium acetate method, and a specific surface area of 607 m^2/g measured by adsorption of ethylene glycol monoethyl ether (Lombardi et al., 2003). Lower than 2 μm fraction was extracted from bentonite by centrifugation. In order to obtain a calcium montmorillonite, 2 g of this fraction was dispersed in 50 mL of 1N CaCl$_2$ solution during 24 h. After this, the dispersion was centrifuged at 8000 rpm during 30 min. The supernatant was removed and the solid was washed until negative

chloride assay. The calcium montmorillonite was dried at 40 °C, ground and stored.

Tetracycline and minocycline were used as hydrochloride; their structural formulas are given in Fig. 1.

TC and MC present different ionic states depending on the solution pH. The tetracycline molecule has three dissociable protons at pH between 2 and 10.5 (Leeson et al., 1963). The fully protonated species of TC (TCH$_3^+$) which exists at pH<3 is shown in Fig. 1. As the pH increases, the first deprotonation step (pKa = 3.3) occurs at the hydroxyl group on C3 leading to the formation of a zwitterion (TCH$_2^\pm$) with a positive charge located on the protonated dimethylamonium group and a negative charge delocalized over the A ring. The second deprotonation step (pKa = 7.7) takes place in the diketone system involving O11 and O12 generating a species with negative net charge (TCH$^-$). Finally, the third deprotonation (pKa = 9.5) involves the dimethylamino group giving rise to species with two negative charges (TC^{2-}).

Minocycline has four pKa values (Orti et al., 2000). The fully protonated species of MC which exists at low pH values (MCH$_4^{2+}$) is shown in Fig. 1. As the pH increases, the first deprotonation step (pKa = 2.8) occurs at the hydroxyl group on C3 leading to the formation of a species with one positive charge (MCH$_3^+$). The second deprotonation step (pKa = 5.0) takes place in the aromatic amino group generating a zwitterionic species (MCH$_2^\pm$). The third deprotonation (pKa = 7.8) involves the O10–O12 ketophenolic hydroxyl group giving rise to a species with negative charge (MCH$^-$). Finally, the fourth deprotonation (pKa = 9.5) involves the dimethylamino group giving rise to a species with two negative charges (MC^{2-}).

The chromophores present in TC and MC molecules are the tricarbonyl system of the A ring and the BCD system, which present UV–vis absorption bands at 275 and 357 nm respectively for TC (Mitscher, 1978) and 269 and 346 nm respectively for MC. As pH increases, a bathochromic shift in the longer wavelength bands is observed. This behavior was explained in terms of different species and conformations in solution (Smyth et al., 1978).
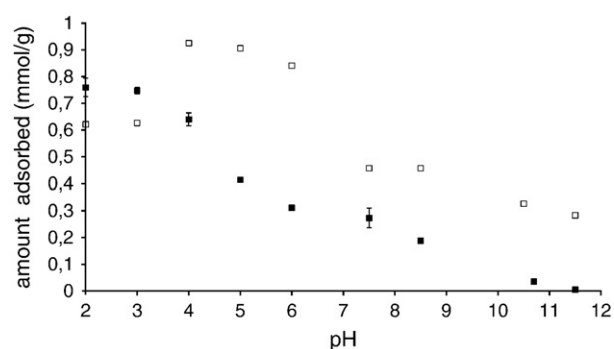

Fig. 2. Effect of pH on TC adsorption (■) and MC adsorption (□) by montmorillonite in 0.01 M NaCl.

Antibiotic stock solutions were prepared just before use to avoid degradation caused by oxygen and light (Oka et al., 1989). NaCl was used for ionic strength adjustment and NaOH or HCl were used for pH adjustment.

2.2. Adsorption studies

Adsorption studies at constant ionic strength (0.01 M NaCl), antibiotics concentration (0.76 mmol/L) and varying pH were performed. A series of 10 mL centrifuge tubes were filled with 3.8 mL of 0.01 M NaCl. Then, 0.2 mL of a stock TC or MC solution (1.336 g/L) having variable pH was added and 4 mL of a 900 mg/L of the montmorillonite dispersion of the desired pH in 0.01 M NaCl solution was added. The pH was readjusted with NaOH and HCl solutions if necessary. The tubes were closed and shaken in darkness during 24 h. Preliminary experiments showed that this time was enough to reach equilibration. After that, the dispersions were centrifuged in a Sorvall RC 5C centrifuge at 8000 rpm during 30 min and the supernatants were removed for UV–vis analysis at the maximum wavelength corresponding to BCD chromophore of each molecule according to the medium pH. The amount of adsorbed TC or MC was calculated as the differences between the initial TC or MC concentration and the concentration that remained in the supernatant. The pH was adjusted to values between 2 and 11, and the measurements were done in triplicate.

2.3. X-ray diffraction

Samples were prepared from montmorillonite, TC-montmorillonite and MC-montmorillonite dispersions at pH 3 and 7. In all cases the clay mineral concentration was 15 mg/mL and the supporting electrolyte was 0.01 M NaCl. The dispersions were deposited and oriented onto glass slides under constant relative humidity conditions of 36%. XRD patterns were obtained using a RIGAKU® Geigerflex X-ray diffractometer with CuKα radiation at 20 mA and 40 kV. Scans were recorded between 2° and 40° (2θ) with a step size of 0.05° and scanning speed of 2°/min.
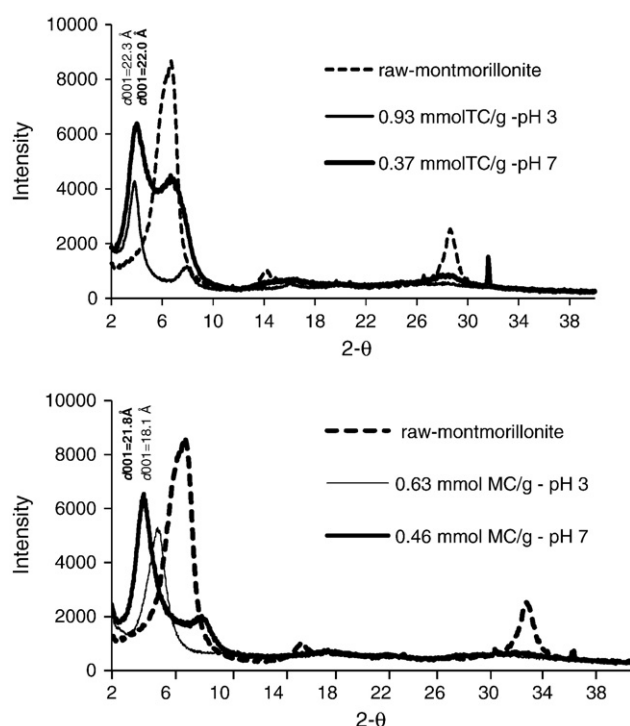

Fig. 3. XRD patterns of montmorillonite (dashed line) and TC and MC-montmorillonite (solid line) prepared at pH 3 and 7 in 0.01 M NaCl.
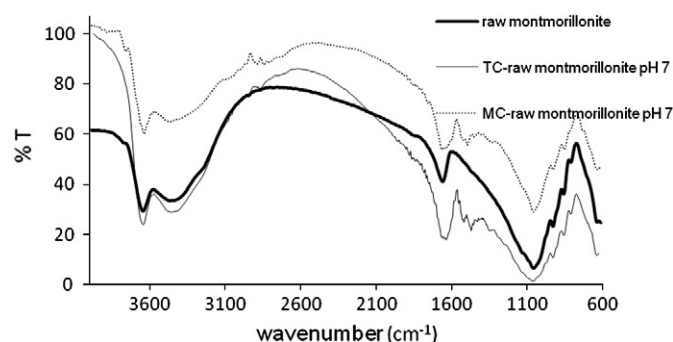

Fig. 4. IR spectra of montmorillonite, TC-montmorillonite and MC-montmorillonite at pH 7.

2.4. IR spectroscopy

Montmorillonite, TC-montmorillonite and MC-montmorillonite (170 mmol/100 g montmorillonite) at pH 7 were analyzed on a Buck 500 spectrophotometer equipped with a deuterated L-alanine doped triglycine sulfate (DLATGS) detector. Infrared spectra were recorder over the region 600–4000 cm^{-1}. The samples were examined in KBr pellets (3 mg/300 mg KBr). Physical mixtures of TC-montmorillonite and MC-montmorillonite powders with the same adsorptive–adsorbent relationship than the prepared complexes were also analyzed for comparison.

2.5. Desorption studies

TC desorption from TC-montmorillonite was studied under different pH conditions. 160 mL of TC-montmorillonite in 0.01 M NaCl solution was prepared from a montmorillonite dispersion (900 mg/L) and a stock TC solution (0.76 mmol/L). After shaking for 24 h, the tubes were centrifuged at 8000 rpm during 30 min and the supernatant was removed. The amount of TC adsorbed was calculated by UV–vis spectroscopy. The TC-montmorillonite was then redispersed in 0.01 M NaCl at the desired pH, to start desorption. The dispersions were kept at 20 °C and constantly shaken in darkness. At different time intervals (from 1 to 80 h) 5 mL dispersion was withdrawn and centrifuged at 8000 rpm during 30 min. To determine the amount of desorbed TC, the TC concentration in the supernatant was measured. All desorption measurements were performed in duplicate.

2.6. Evaluation of antibacterial activity

TC-montmorillonite and MC-montmorillonite antibacterial activity were tested with E. coli ATCC 25922 by the test of susceptibility on solid medium.

2.6.1. Preparation of bacterial suspensions

E. coli culture was prepared from streaks on a nutrient agar slant. A sterile wire loop was used to take a small sample of bacteria from the agar. The bacterial sample was placed into a tube containing 4 mL of sterile distilled water and conveniently diluted to reach a concentration of 10^6 bacteria/mL.

Table 1
Effect of pH on TC desorption.

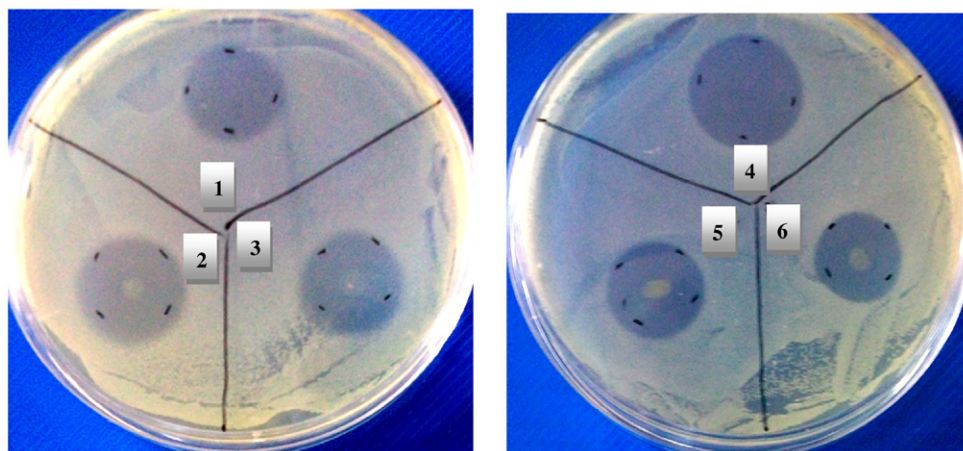| System	pH	% Desorption
TC-raw montmorillonite	3	15
TC-raw montmorillonite	5	85
TC-raw montmorillonite	7	100
TC-Ca montmorillonite	4	27
TC-Ca montmorillonite	7	51

Fig. 5. Zone of inhibition (ZOI) test: TC solution (1), TC-raw montmorillonite (2), TC–Ca montmorillonite (3), MC solution (4), MC-raw montmorillonite (5), MC–Ca montmorillonite.

2.6.2. Dispersion preparation of the adsorption complexes

Dispersions of TC-raw montmorillonite (0.76 mmol/g), TC-calcium montmorillonite (0.46 mmol/g), MC-raw montmorillonite (0.71 mmol/g) and MC-calcium montmorillonite (0.68 mmol/g) were prepared as shown in Section 2.5. The resulting pH of the dispersions was approximately 4.

The TC and MC-montmorillonites were washed three times with 40 mL of aqueous 0.01 M NaCl solution using high agitation magnetic stirring for 30 min. The dispersions were centrifuged at 9000 rpm during 15 min and the washed adsorption complexes were redispersed in 0.01 M NaCl to obtain an antibiotic concentration of 1.33 mg/mL. 3 μL of TC-montmorillonite or MC-montmorillonite dispersions were collected for Zone of inhibition (ZOI) test and the process was repeated one additional time.

2.6.3. Zone of inhibition test

Petri plates containing 20 mL of the solidified Muller Hinton were inoculated with 0.1 mL of the suspension of 10^6 bacteria/mL of *E. coli* ATCC 25922 spread with a sterile Digralsky spatula. Once the inoculum was dry, 3 μL of TC or MC solution (1.33 mg/mL) or 3 μL of washed TC and MC-montmorillonite dispersions was added. The plates were then incubated at 37 °C for 24 h and then the inhibition zone diameter was measured. The determinations were made for each antibiotic and its corresponding complexes by duplicate. A montmorillonite dispersion (900 mg/L) without added antibiotics was analyzed in the agar plate to evaluate possible antibacterial activities.

3. Results and discussion

3.1. Adsorption studies

The effect of the pH on TC and MC adsorption on raw montmorillonite is illustrated in Fig. 2.

The adsorption of both antibiotics was high at low pH and decreased with increasing pH. Similar pH effects were reported for the adsorption of TC on several clay minerals (Browne et al., 1980).

Table 2
Zone of inhibition test, data are mean values.

Samples	Inhibition zone diameter (mm)
TC solution	20.0
TC-raw montmorillonite	19.0
TC-Ca montmorillonite	18.0
MC solution	20.0
MC-raw montmorillonite	17.2
MC-Ca montmorillonite	16.0

At pH values from 2 to 3, MC forms a divalent cation whereas TC is mainly present as a monovalent cation. Due to the formation of a divalent cation, MC adsorption was slightly lower than TC adsorption (0.63 mmol/100 g and 0.75 mmol/100 g respectively). At pH>4 the adsorption of MC was higher than that of TC. In addition, at pH>10 the adsorption of TC was negligible whereas the adsorption of MC was significant (0.30 mmol/g).

According to Browne et al. (1980), the effects of pH on the adsorption of TC are due to the interaction between montmorillonite and the cationic species TCH_3^+ (pKa = 3.3), whose concentration decreases with increasing pH. Parolo et al. (2008b) suggested that the adsorbed species were not only TCH_3^+ but also TCH_2 and TCH^-. The adsorption decreased in the following order: $TCH_3^+ > TCH_2 > TCH^-$ (Parolo et al., 2008b). Li et al. (2010) provided a detailed balance between TC adsorbed and exchangeable cations desorbed, suggesting that cation exchange was the main mechanism for TC adsorption on montmorillonite.

The electrostatic attraction may play an important role in the adsorption of organic substances on montmorillonite (Lagaly et al., 2006) but there may also exist non-electrostatic interactions between the organic species and the montmorillonite surface. These interactions, such as hydrogen bonding and van der Waals interaction are likely to operate not only with cationic species (TCH_3^+, MCH_4^{2+}, MCH_3^+) but also with TCH_2, TCH^-, MCH_2^-, and MCH^- and they will contribute to the adsorption.

Neutral TC and MC species or those with a net negative charge present the protonated trialquilamine functional group in their structure (pKa = 9.5). At high pH values species with two negative charges (TC^{2-} and MC^{2-}) are predominant. This would account for the almost null retention of TC^{2-} on the negative clay mineral. However, MC^{2-} species were adsorbed in an amount of 30% of the CEC. Likely, the second trialquilamine group at C7 of the MC structure can promote interactions with the mineral surface.

3.2. X-ray diffraction

The XRD diagrams of the adsorption complexes of TC-raw montmorillonite and MC-raw montmorillonite at pH 3 and 7 are shown in Fig. 3, together with the diagram corresponding to the montmorillonite dispersion (15 mg/mL) in 0.01 M NaCl.

For the raw montmorillonite dispersed in the supporting electrolyte a broad 001 reflection was observed at 6.60° 2θ (d = 13.4 Å). The deconvolution algorithm using the Gaussian method showed the contribution of two reflections at 92 and 6.90° 2θ (d = 14.9 and 12.8 Å) indicating the coexistence of two hydration states of the sodium montmorillonite (Brigatti et al., 2006; Pantanetti et al., 2008).

When the montmorillonite was dispersed in water, the 001 reflection at 7.00° 2θ ($d = 12.6$ Å) indicated the monolayer hydrate.

TC-montmorillonite at pH 3 (0.93 mmol/g) produced a diffraction pattern with a first order reflection with $d = 22.3$ Å and a second order one with $d = 11.2$ Å. This basal spacing evidences the intercalation of TC indicating TC monolayers (Parolo et al., 2008b).

TC-montmorillonite at pH 3 (0.37 mmol/g) showed two reflections located at 4.10° 2θ ($d = 22.0$ Å) that corresponded to TC intercalation and at 6.90° 2θ ($d = 13.2$ Å) corresponding to the hydrated sodium montmorillonite.

The diagrams of MC-montmorillonite at pH 3 (Fig. 3) showed a 001 reflection at 4.88° 2θ ($d = 18.1$ Å) and the 002 reflection at 9.94° 2θ ($d = 8.9$ Å); whereas at pH 7 these reflections appeared at 4.06° 2θ ($d = 21.8$ Å) and 7.68° 2θ ($d = 11.5$ Å). The results indicate the intercalation of a monolayer of MC molecules and pH-dependent orientations. For pH 3 cationic species (MCH_4^{2+} and MCH_3^+) are predominant and the positively charged groups are parallel to the basal plane. When the predominant species is the zwitterion (pH = 7), the molecule is oriented in such a way that the positively charged groups are closer to the surface whereas the negatively charged groups move away from the surface.

3.3. IR spectroscopy

The main bands of montmorillonite (Fig. 4) were: 3639 cm^{-1} attributed to the –OH stretching vibration of structural hydroxyl groups, 3434 cm^{-1} and 1634 cm^{-1} assigned to the –OH stretching and –OH deformation vibration of water respectively, and 1062 cm^{-1} assigned to Si–O stretching vibration (Madejová and Komadel, 2001).

The spectra of the physical mixtures of TC and MC with montmorillonite (spectra not shown) presented the bands corresponding to montmorillonite as well as those attributed to the adsorptive: 2760 cm^{-1} and 2670 cm^{-1} associated to –NHR$_3^+$ group, 1670 cm^{-1} and 1526 cm^{-1} assigned to the Amide I and Amide II bands, 1618 cm^{-1} and 1586 cm^{-1} assigned to C=O stretching vibration at rings A and C respectively, 1466 cm^{-1} assigned to C–C stretching vibration, 1380 cm^{-1} assigned to –CH$_3$ deformation vibration and 1244 cm^{-1} which is attributed to C–N amine stretching vibration (Caminati et al., 2002). As MC presents a Ar–NHR$_2^+$ group, differences in the IR spectrum at 2700–3000 cm^{-1} were observed, corresponding to the –CH$_3$ and NHR$_3^+$ stretching vibrations.

Montmorillonite bands were observed in the IR spectra of TC and MC-montmorillonite, whereas the bands assigned to NHR$_3^+$ group were not observed. The bands corresponding to –CH$_3$ and C–C were not changed in relation to the spectrum of bulk TC and MC. The bands at 1670 and 1526 cm^{-1} assigned to the amide group were shifted by 20 cm^{-1} to lower frequencies with respect to bulk TC. Similar results were found for TC-montmorillonite (Porubcan et al., 1978; Chang et al., 2009b).

The results confirm that the NHR$_3^+$ groups of TC and MC are bound to the clay mineral surface. Whereas other polar groups of the molecule could participate in non-electrostatic interactions with the mineral surface (Parolo et al., 2008a).

3.4. Desorption studies

Table 1 presents the desorption data after 1 h contact of TC-montmorillonite in the electrolyte solution.

TC desorption increased with pH. For TC-raw montmorillonite at pH 7, the desorption was complete whereas for the calcium-exchanged montmorillonites it was approximately 50% thus indicating the high affinity between TC and calcium. TCH$^-$ species at pH 7, with the diketone system involving O11 and O12 of BCD rings, act as electron donor species interacting with calcium ions (Mitscher et al., 1972).

3.5. Antibacterial activity

The antibiogram tests are shown in Fig. 5 and the inhibition zone diameters (in mm) are given in Table 2. No antibacterial activity was detected for raw and calcium-exchanged montmorillonites. Similar results were reported in literature (Magaña et al., 2008).

TC and MC solutions and all montmorillonite complexes had the same antibiotic concentrations. The antibacterial activities of TC and MC solutions were identical (20.0 mm) whereas the inhibition diameters (19.0 and 18.0 mm for TC–Na and Ca montmorillonite; 17.2 and 16.0 mm for MC–Na and Ca montmorillonite respectively), were slightly lower than those of TC and MC solutions. Thus, the TC and MC-montmorillonites had good antibacterial activity, which indicates that TC and MC are desorbed from the clay mineral and diffuse into the agar plate. Similar results were found with norfloxacin adsorbed on fluoromicas (Dizman et al., 2007).

TC and MC spread on the agar plate that contains sodium chloride and other organic compounds which could facilitate the exchange of cations on the montmorillonite surface in the reverse direction.

4. Conclusions

The raw (sodium) montmorillonite, which was previously shown to be a pharmaceutically adequate material, adsorbed MC and TC depending on the pH. At low pH values, where TC and MC are predominant cationic species, retention was high.

XRD and IR data showed the intercalation of TC and MC into montmorillonite.

The antimicrobial inhibition shown by MC and TC was slightly reduced by their adsorption on raw or calcium montmorillonite. Montmorillonite did not show any antibacterial property. TC and MC desorbed from the clay mineral surface and then diffused by the culture medium.

The excellent activity shown by the TC and MC-montmorillonite appears as a promising field of application, in particular for the formulation of topical products.

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