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International Journal of Pharmaceutics 332 (2007) 24–30
INTERNATIONAL JOURNAL OF PHARMACEUTICS
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Rapid communication

Bismuth-norfloxacin complex: Synthesis, physicochemical and antimicrobial evaluation

Anwar R. Shaikh, Rajani Giridhar, Mange Ram Yadav *

Pharmacy Department, Faculty of Technology and Engineering, The M.S. University of Baroda, Vadodara 390 001, India

Received 24 August 2006; received in revised form 14 November 2006; accepted 15 November 2006
Available online 19 November 2006

Abstract

Norfloxacin is a fluoroquinolone antibacterial agent which is active against various Gram-positive as well as Gram-negative microorganisms. Presence of metal ions considerably alters the activity of fluoroquinolones against potentially susceptible bacteria. As bismuth is known to possess a good antibacterial activity, bismuth complex of norfloxacin was prepared by reacting bismuth citrate with aqueous solution of norfloxacin. The structure of the bismuth-norfloxacin complex (BNC) was confirmed by spectral, chemical and elemental analysis. Antimicrobial studies were carried out using agar diffusion method against *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (NTCC 10320), *Staphylococcus aureus* (ATCC 29213), *Bacillus pumilis* (NTCC 8241) and *Staphylococcus epidermidis* (ATCC 12228). The results showed significant increase ($p < 0.05$, Tukeys test) in antibacterial activity of BNC as compared with norfloxacin and physical mixture of norfloxacin and bismuth citrate. This increase in activity is being considered due to increased bioavailability of the metal drug complex. Thus, the use of the BNC may be preferable over norfloxacin alone.

Keywords: Bismuth-norfloxacin complex; Antibacterial activity; Norfloxacin

1. Introduction

Fluoroquinolones are broad spectrum antibiotics widely used for the treatment of numerous diseases (Reynolds, 1993; Naumann and Dopp, 1989). Norfloxacin (1-ethyl-6-fluoro-4-oxo-7-(1-piperazinyl)-l,4-dihydroquinoline-3-carboxyli acid) is a widely used representative member of this family. In general, it is active against a wide variety of aerobic Gram-negative and Gram-positive bacteria but specifically, active against aminoglycoside-resistant *Pseudomonas aeroginosa* and beta-lactamase producing organisms. The mechanism of action of norfloxacin involves inhibition of bacterial DNA gyrase which is essential for DNA replication (Takahata and Nishino, 1988; Shen, 1989). It has been proposed that metal complex intermediates are involved in this process (Shen et al., 1989; Shen and Pernet, 1985). The ligand environment of transition metals (present in very low concentration *in vivo*) can be considerably altered upon the administration of a therapeutically effective dose of an antibacterial drug. This change in balance between the metal ion and the ligand may have a profound effect upon the activity of drug against potentially susceptible bacteria. It has also been reported that the transport of organic ligands into the cells can be facilitated by the formation of metal complexes (Gao et al., 1995). Bismuth is known to possess good antibacterial activity (Clitherow, 1991; Peter and Colm, 2005). Ranitidine-bismuth citrate complex is clinically available. Transport of quinolones across the bacterial cytoplasmic membrane is strongly pH dependent, peaking at neutral pH (Furet et al., 1992). It has been proposed that zwitterionic and uncharged quinolone species are responsible for diffusion through cytoplasmic membranes (Kawai and Matsubayashi, 1996) and the presence of metal ions results in a higher uptake of quinolones by bacterial cells compared to that of the drug alone (Ma et al., 1997). Therefore, the formation of metal complexes may increase the bioavalability of the metal ion or the ligand or both. It is believed that bismuth in complexed form is more stable and better tolerated (Clitherow, 1991). Hence, it was planned to synthesize and evaluate the bismuth complex of norfloxacin and to evaluate it for its antimicrobial spectrum. Bismuth-norfloxacin complex should have applications in the

* Corresponding author. Tel.: +91 265 2794051; fax: +91 265 2423898.
E-mail address: mryadav11@yahoo.co.in (M.R. Yadav).

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doi:10.1016/j.ijpharm.2006.11.037

treatment of peptic ulcers caused by *H. pylori* and conjunctivitis caused by bacterial infections in the eye.

2. Materials and methods

2.1. Materials

Norfloxacin was supplied by Alembic Ltd. and Mercury Laboratories, Vadodara India, as gift samples. Bismuth oxynitrate, citric acid, dimethyl sulphoxide (DMSO) and ammonia solution were obtained from s.d. fine chemicals, Mumbai, India. Muller Hinton agar and nutrient broth were obtained from HiMedia Ltd. Mumbai. *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (NTCC 10320), *Staphylococcus aureus* (ATCC 29213), *Bacillus pumilis* (NTCC 8241), *Staphylococcus epidermidis* (ATCC 12228) obtained from Food and Drug Laboratory (FDL) Vadodara, India, were used for antimicrobial screening.

2.2. Preparation of bismuth complex of norfloxacin

Bismuth citrate was prepared following the reported procedure (Clitherow, 1991) by adding bismuth oxynitrate (5.74 g, 0.02 mol) to a solution of citric acid (8.40 g, 0.043 mol) in water (80 ml). This mixture was heated on steam bath with frequent stirring for 30 min. The precipitate obtained was washed with water and dried under vacuum to give bismuth citrate (yield: 6.50 g, 81% m.p., 325–330 °C (reported 330 °C (Aldrich Catalogue, 2001)). Analysis found: C, 18.08; H, 1.34; Bi, 52.00 $C_6H_5BiO_7$, calculated: C, 18.01; H, 1.32; Bi, 52.20.

Norfloxacin (0.802 g, 0.00251 mol) was added to a solution of bismuth citrate (0.2 g, 0.00502 mol) in dilute hydrochloric acid (5 ml, 5%) with constant stirring. This resulting solution was basified with strong ammonia solution drop by drop and was heated on steam bath for 5 h. The precipitate was filtered, washed with distilled water and dried under vacuum to a constant weight (yield: 0.92 g, 85%, m.p. 280 °C (dec.)). Analysis found C, 50.89; H, 4.92; N, 11.04; Bi, 13.80. $(C_{16}H_{18}FN_3O_3)_4Bi{\cdot}2H_2O$, calculated: C, 50.63; H, 4.78; N, 11.07; Bi, 13.76.

2.3. Analysis of bismuth-norfloxacin complex

Infrared (IR) spectra were obtained on Schimadzu FTIR 8300 using KBr pellets. Ultraviolet spectra were obtained on Schimadzu 1700. Elemental analysis was performed on Thermo Finnigan FLASH EA 1112 Series. Bismuth content was determined using Thermo S-series atomic absorption spectrometer. The pH measurements were carried out with a control dynamic pH meter. Karl-Fisher titrimeter (Aqua Cal) was used for the determination of water content. Differential scanning calorimetery (DSC) was conducted on Schimadzu DSC 60 apparatus. The sample size used for DSC was about 5 mg. The scanning speed was kept at 10 °C/min. Decomposition temperature of the complex was determined by thermal analysis, using a Perkin-Elmer TGA-7 thermobalance operating at a heating rate of 10 °C/min.

2.4. Karl-Fisher titrimetry for water content

Karl-Fisher titration was performed in order to find the number of bonded water molecules. Karl-Fisher reagent was calibrated with disodium tartarate. An accurately weighed amount of compound was added to the dry methanol in a KF reaction vessel and titrated against Karl-Fischer reagent. Titration was performed in triplicate to get reproducible results. The percentage water content was determined from Eq. (1):

$$\%\ \text{moisture content} = \frac{V \times \text{water factor} \times 100}{W \times 1000} \quad (1)$$

where water factor was obtained by titrating known amount of water sample with Karl-Fischer reagent, '*V*' represents milliliters of Karl-Fischer reagent required for complete neutralization of water in the sample and '*W*' represents weight of sample in grams.

2.5. Molar absorbtivity determination

To find out the number of moles of ligand (norfloxacin) attached to the central metal atom (bismuth), molar absorbtivity of the compound was determined. Accurately weighed amount of compound was dissolved in methanol and absorbance was recorded on a UV spectrophotometer at its λ_{max} 277.5 nm. Molar absorbtivity as obtained for the synthesized compound was compared with the molar absobtivity of the ligand to get the mole ratio of metal: ligand equal to 1:3.96 (calculated 1:4). Molar absortivity was determined from Eq. (2):

$$\text{molar absorbtivity} = \frac{A}{W} \times \text{dilution factor} \times \text{molecular weight of compound} \quad (2)$$

where *A* is the absorbance and *W* represents weight of sample in milligrams.

2.6. Effect of pH upon complexation of bismuth with norfloxacin

The complexation of metals with quinolone in aqueous solution depends mainly upon pH. To investigate the pH dependence of the complexation between bismuth and the quinolone derivative norfloxacin, UV–vis spectroscopy was used. The solutions norfloxacin (0.0005 mol/l) and mixture of norfloxacin (0.0005 mol/l) and bismuth citrate (0.000125 mol/l) were prepared in 0.1N HCl. In both solutions, pH was adjusted by dilute ammonia solution (10%) to get solution of pH 2.1, 4.3, 7.0, 8.3 and 10.0. A UV–visible spectrum was recorded for all of the solutions in the range 200–400 nm.

2.7. pH-solubility studies of norfloxacin and its bismuth complex

Solubilities were determined for norfloxacin and for BNC at 37 °C as a function of pH in buffer solutions varying in pH from 2 to 8. The buffer solutions were constituted from

hydrochloric acid (0.2 M), sodium hydroxide (0.2 M), disodium hydrogen phosphate (0.2 M), potassium dihydrogen phosphate (0.2 M) (Indian Pharmacopoeia, 1996). An excess of the compound was added to 10 ml of each buffer solution and agitated at 37 °C for 24 h. This was then filtered immediately in test tubes maintained at 37 °C, and the equilibrium pH of the filtrate was recorded. The samples (in triplicate) after appropriate dilutions with respective buffer pH were analyzed by ultraviolet spectroscopy (Sharma et al., 2003) using a standard plot of norfloxacin in the same medium (pH 2.0, $r^2 = 0.999$, pH 4.0, $r^2 = 0.998$, pH 6.5, $r^2 = 0.976$, pH 8.0, $r^2 = 0.998$ by UV spectrophotometer at 277 nm) and for BNC (pH 2.0, $r^2 = 0.991$, pH 4.0, $r^2 = 0.998$, pH 6.5, $r^2 = 0.998$, pH 8.0, $r^2 = 0.998$ by UV spectrophotometer at 271 nm).

2.8. Antibacterial activity assay

The minimum inhibitory concentration (MIC) of norfloxacin as well as synthesized compound was determined by agar diffusion method using Muller Hinton agar as described by the National Committee for Clinical Laboratory Standards in its guidelines (NCCL Guidelines, 1993; United States Pharmacopoeia, 2002). Samples were initially dissolved in DMSO at concentrations between 0.0125 and 128 mg/l. Isolates were grown for 24 h in nutrient broth to provide a turbidity of approximately 10^9 cfu/ml. Bacterial suspensions were diluted with soft agar containing tubes at 45–50 °C. These soft agar tubes were then poured over the Muller Hinton agar plates previously prepared and allowed to solidify under laminar flow for 15 min. Filter paper discs, previously sterilized were, placed over the agar plates containing bacterial suspensions. Sterile pipettes (0.2 ml) were used in aseptic conditions to add the compounds on filter paper disc. Sample solution (0.15 ml) was added to each disc. Same volume of the control (DMSO) was also added on one of the other disc in each plate. The plates were then placed in an incubator at 37 °C within 15 min of addition of the compounds on the filter paper disc. After 18–24 h of incubation, the plates were examined and the diameter of zones of complete inhibition was measured by zone diameter measuring scale (HiMedia).

3. Results and discussion

3.1. Characterization of bismuth-norfloxacin complex

The structure of norfloxacin as well as BNC is shown in Fig. 1. The IR spectra of quinolones are most representative in the region 1800–1300 cm^{-1} (Leban et al., 1997; Bukovec et al., 1997). The proposed structure of BNC was confirmed by its analytical data. The IR spectrum of norfloxacin (Fig. 2a) showed two characteristic peaks at 1716 cm^{-1} (due to –COOH stretching) and 1631 cm^{-1} (pyridone keto). BNC showed absence of peak at 1716 cm^{-1} due to deprotonation of –COOH group and formation of Bi–O bond (Fig. 2b). The peak at 1631 cm^{-1} was retained in the IR spectrum of BNC. Peaks at 1558 and 1456 cm^{-1} in case of BNC correspond to asymmetrical and symmetrical carboxylate anion stretching, respectively. The spectrum is generally very similar to that of the copper-ciprofloxacin complex (Turel et al., 1994; Turel et al., 1996) where the copper ion is coordinated with the carbonyl oxygens of quinolone molecule. Atomic absorption spectroscopy and elemental analysis of the hydrated BNC showed it to possess a ligand–metal ratio of 4:1 with two molecules of water which was further confirmed by Karl-Fischer titration which gave the water content equal to 2.10% (calculated for $2H_2O$: 2.37%). The ligand to metal ratio was further confirmed by determination of molar absorbtivity spectrophotometrically (metal:ligand ratio-calculated 1:4, found 1:3.96).

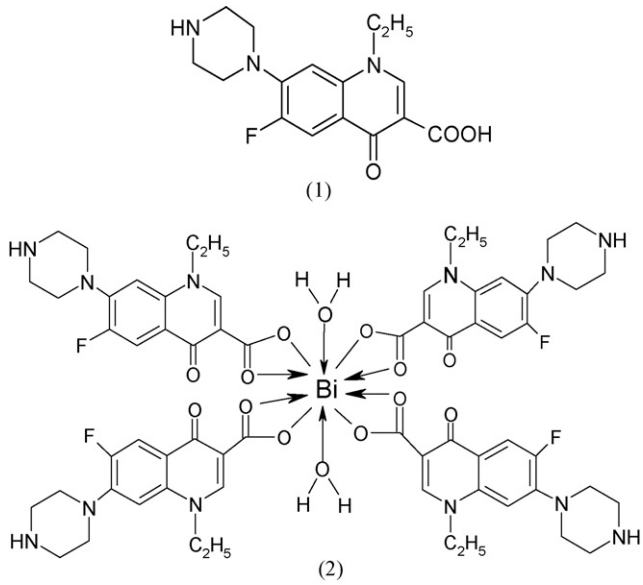

Fig. 1. Structure of norfloxacin (1) and bismuth-norfloxacin complex (2).

Analysis calculated for $(C_{16}H_{18}FN_3O_3)_4Bi{\cdot}2H_2O$: C, 50.63; H, 4.78; N, 11.07; Bi, 13.76; found C, 50.89; H, 4.92; N, 11.04; Bi, 13.80. The thermal curves obtained for norfloxacin, bismuth citrate, physical admixture of norfloxacin with bismuth citrate and BNC are shown in Fig. 3. The endothermic peak of the parent drug at 223.5 °C (Fig. 3a), as well as the exothermic peak at 330.2 °C of bismuth citrate (Fig. 3b) though present in its physical mixture (Fig. 3c) at or around the same temperature, disappears completely in the case of its bismuth complex with the appearance of new exothermic peak at 280.45 °C (Fig. 3d), thus corroborating the formation of a new compound. The peak at 280.45 °C appears as broad and exothermic, it might be due to melting with decomposition and loss of water of crystallization of the complex. A broader exotherm indicate a slow change in heat capacity and indicate behaviors like dehydration. (Willard et al., 1986). Thermal gravimetric curve shows that the pyrolysis of compound begins at 275 °C and ends at 360 °C (Fig. 4).

3.2. Physicochemical evaluation of BNC

3.2.1. Effect of pH upon complexation of bismuth with norfloxacin

UV–visible spectroscopy was used to investigate the pH dependence of the complexation of bismuth with norfloxacin

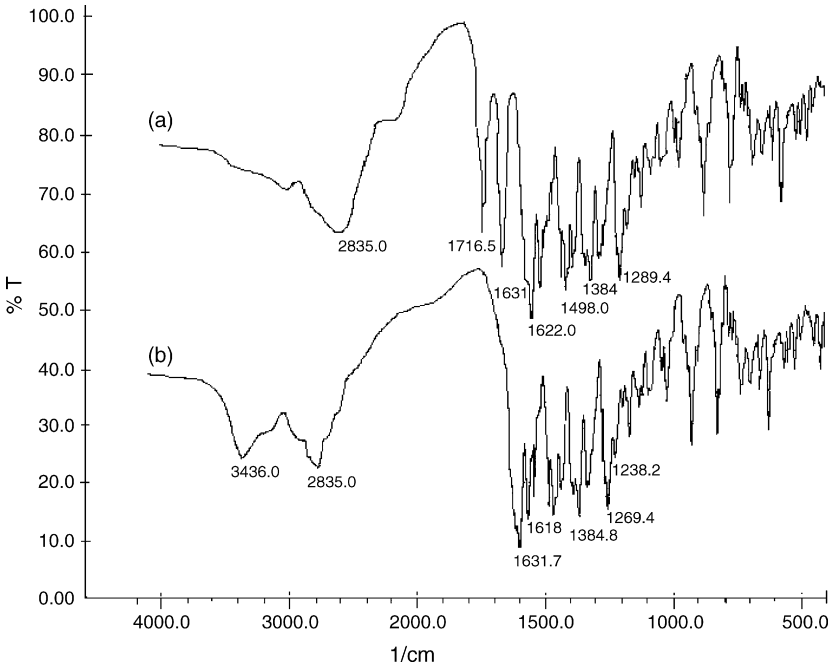

Fig. 2. IR spectra of norfloxacin (a) and bismuth-norfloxacin complex (b).

in aqueous solution. The absorption maxima for an aqueous solution of norfloxacin at pH 2.1 appeared at 277 and 316 nm. On increasing the pH, the maximum at 277 nm shifted to lower values and the maxima at 316 nm shifted to higher value. These changes can be attributed to the extent of ionization of the carboxylic group as a consequence of the removal of a proton (Borrel and Montero, 1997). At pH ≥ 7, the absorption maxima appeared at 224, 271, 322 nm in case of norfloxacin. These

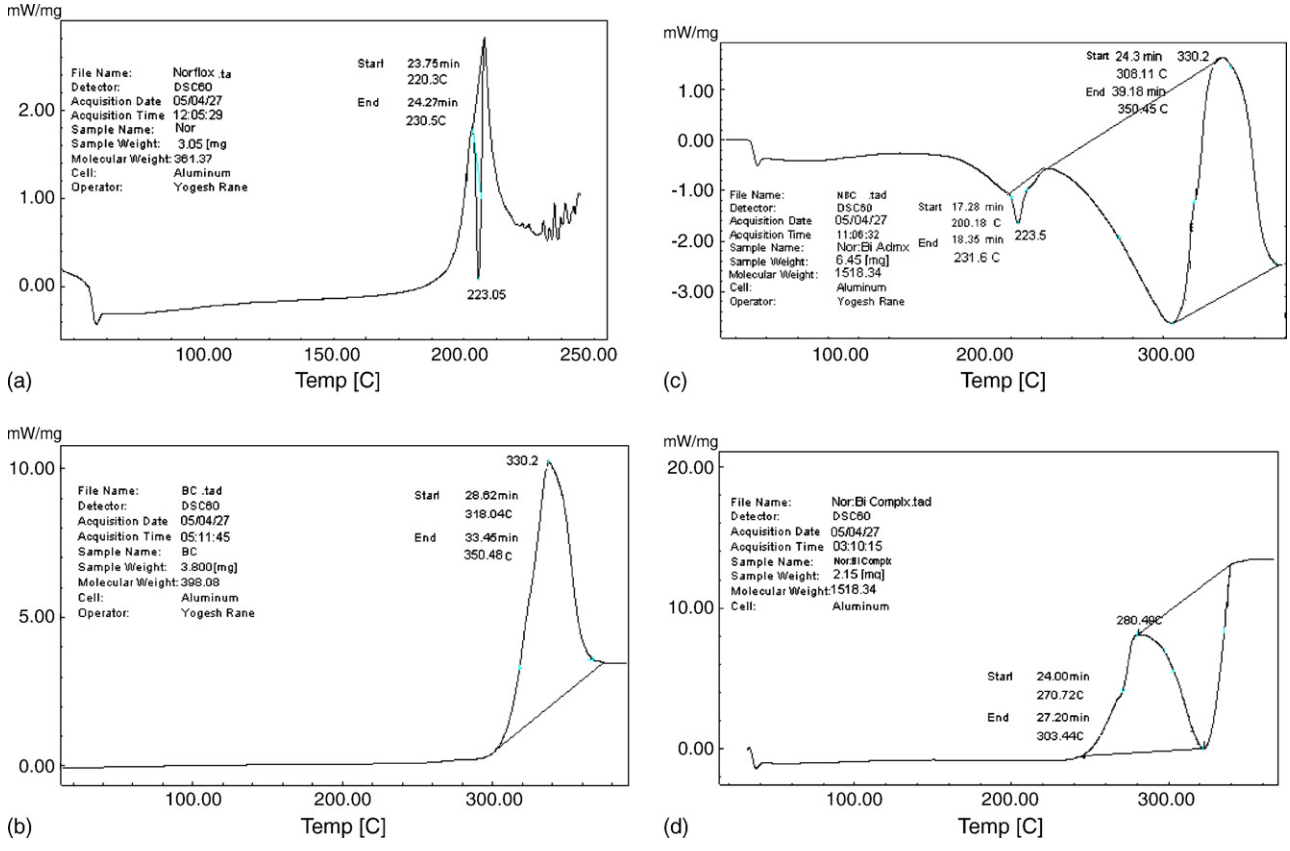

Fig. 3. DSC curves of norfloxacin (a) bismuth citrate (b); physical admixture of norfloxacin and bismuth citrate(c) and bismuth-norfloxacin complex (d).

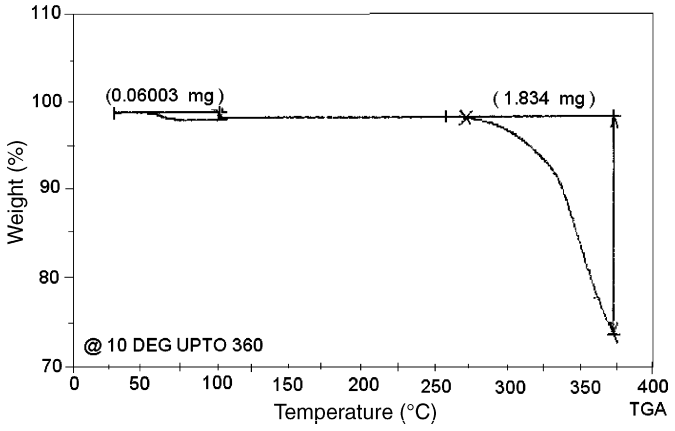

Fig. 4. Thermogravimetric (TGA) graph for bismuth-norfloxacin complex.

Table 1
Effect of pH upon complexation of bismuth with norfloxacin

pH	Absorption maxima (λ_{max}) values (nm)	
	Bismuth citrate and norfloxacin	Norfloxacin
2.1	277, 314	277, 314
4.3	276, 315	276, 315
6.2	275, 323	274, 322
7.0	223, 271, 323	224, 271, 324
8.3	275, 318	226, 271, 324
10.0	275, 320	271.5, 324

spectra were compared with those of a solution of bismuth citrate and norfloxacin at the same pH values. At pH 2–6, no significant differences were observed between the spectra of the two solutions (Table 1), indicating no interaction between bismuth ions and the norfloxacin. At pH > 7, both bathochromic and hypsochromic shifts were found in the spectra with the band of norfloxacin at 272 nm, shifting to 275 nm, in the presence of bismuth ions (Fig. 5). Thus, it is proposed that at pH above 7, there is complexation between bismuth ions and norfloxacin.

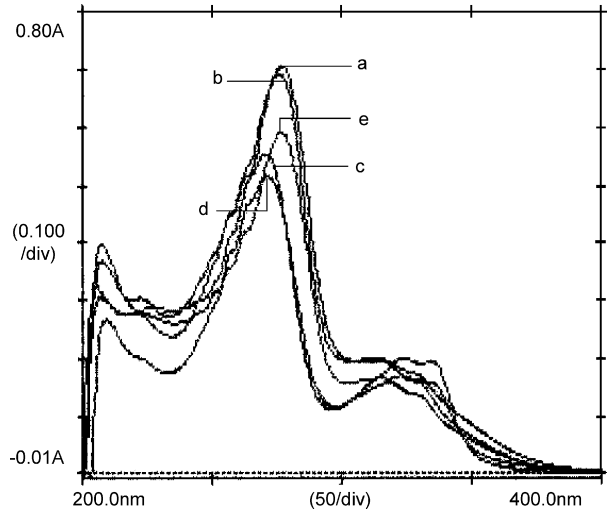

Fig. 5. UV absorption spectra of norfloxacin as a function of pH—a: pH 2.1, b: pH 4.3, c: pH 7.0, d: pH 8.3 and BNC and e: BNC at pH 8.3.

Table 2
Solubility of norfloxacin and BNC at different pH

pH	Solubility (mg/ml)	
	Norfloxacin	BNC
2.0	5.329	6.431
4.0	4.087	5.344
6.5	3.916	4.020
8.0	3.880	2.382

3.2.2. *pH-solubility profile*

The solubility of norfloxacin and its bismuth complex in different pH buffers at 37 °C indicated that the solubility of the BNC was more than that of norfloxacinn till pH 6.5 (Table 2). Above pH 6.5, a significant decrease in the solubility of BNC was observed whereas solubility of norfloxacin did not change significantly. The increased solubility of the BNC as compared to norfloxacin gives the bismuth complex an advantage as antibacterial agent.

3.3. *Antimicrobial evaluation of BNC*

Antibacterial activity was carried out for the synthesized complex as well as the parent drug. Table 3 shows the MIC value for the synthesized compound BNC, norfloxacin and physical admixture of both norfloxacin and bismuth citrate against various Gram-positive as well as Gram-negative micro organisms (Fig. 6). A significant increase in antibacterial activity ($p < 0.05$ Tukeys test, Bolton and Bon, 2001) was observed for BNC. As the MIC value for norfloxacin as well as bismuth citrate norfloxacin physical admixture was similar or nearly the same ($p > 0.05$ Tukeys test), clearly indicating that the complexation with bismuth resulted in a significant increase in the activity of norfloxacin.

Transport of quinolones across the bacterial cytoplasmic membrane is strongly pH dependant (Furet et al., 1992; Zupan et al., 2001). Uncharged quinolone species are responsible for diffusion through cytoplasmic membranes (Kawai and Matsubayashi, 1996). BNC (2), remaining an unionized species (unlike norfloxacin) at neutral or higher pH values would be transported into higher concentration in the microorganisms leading to lowering of MIC of BNC in comparison to nor-

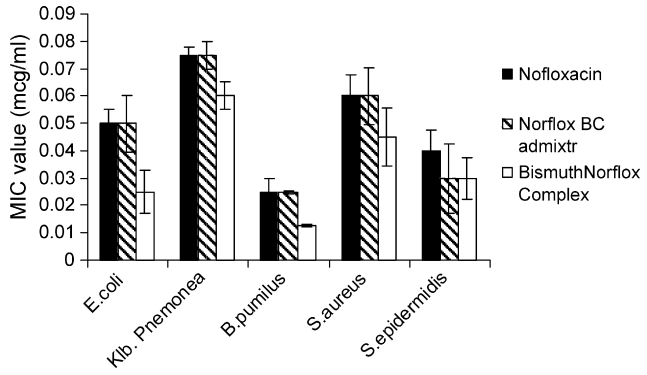

Fig. 6. Comparison of MIC values of the norfloxacin (1), norfloxacin with bismuth citrate in physical admixture and bismuth-norfloxacin complex (2).

Table 3
MIC values (mg/l) for (a) norfloxacin, (b) bismuth-norfloxacin physical admixture and (c) bismuth-norfloxacin complex

Organism used (strain no.)	Minimum inhibitory concentration in mg/l ± standard deviation			*P*-value (Tukeys test)
	Norfloxacin	Norflox. and bismuth citrate physical admixture	BNC	
Escherichia coli (ATCC 25922)	0.050 ± 0.005	0.050 ± 0.010408	0.0250 ± 0.007638	<0.05
Klebsiella pneumoniae (NTCC 10320)	0.075 ± 0.00288	0.075 ± 0.005	0.0600 ± 0.005	<0.01
Bacillus pumilis (NTCC 8241)	0.025 ± 0.005	0.025 ± 0.005	0.0125 ± 0.0005	<0.01
S. aureus (ATCC 29213)	0.060 ± 0.007638	0.060 ± 0.010408	0.0450 ± 0.007638	<0.05
Staphylococcus epidermidis (ATCC 12228)	0.040 ± 0.007638	0.030 ± 0.012583	0.0300 ± 0.007638	<0.05

ATCC: American type culture collection; NTCC: national type culture collection.

floxacin and physical admixture of norfloxacin and bismuth citrate.

A number of studies have indicated the antimicrobial activities of many heavy metals due to their effects on iron uptake by bacteria (Bland et al., 2004). Iron is a co-factor for many essential enzymes (Domenico and Reich, 1996). Potant antimicrobial activity of BNC would result out of a combination of higher transport of the complex (2) through the cell membrane and iron limitation into the cells of the bacteria.

4. Conclusion

Transport of organic ligands into bacterial cells can be facilitated by the formation of metal complexes. Hence, bismuth complex (2) of norfloxacin (1) was synthesized. It was characterized by UV, IR, DSC, atomic absorption spectroscopy, Karl-Fischer titrametry and elemental analysis. The complex was found to possess metal to ligand ratio of 1:4. It has been observed that complexation between bismuth ions and norfloxacin takes place above pH 7. The Solubility of NBC was found to be more than that of norlfoxacin. Agar diffusion method was used for antibacterial activity. BNC was found to possess better activity (lesser MIC value) than that of norfloxacin as well as bismuth citrate and norfloxacin physical admixture. It was concluded that BNC can be a better alternative to norfloxacin as an antibacterial agent.

Acknowledgements

The authors are thankful to Prof. N.S.S Murthy, Metallurgy Department, The M.S. University of Baroda, India for providing atomic absorption spectroscopy facility.

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