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ORIGINAL ARTICLE

Antioxidative mechanisms protect resistant strains of *Staphylococcus aureus* against ciprofloxacin oxidative damage

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ABSTRACT

The aim of this investigation was to determine whether the antioxidant defences protect resistant strains of *Staphylococcus aureus* against ciprofloxacin oxidative damage. Reactive oxygen species (ROS) were determined by chemiluminescence and nitric oxide (NO) was assayed by Griess reaction. The accumulation of ciprofloxacin was examined by fluorometry and oxidation of protein, catalase, ferrous reduction antioxidant potency (FRAP), carbonyls and advanced oxidation protein products (AOPP), studied by spectrophotometry. Ciprofloxacin stimulated higher production of ROS and NO in the susceptible strains than in the resistant ones. There was higher accumulation of antibiotic in sensitive strains than in resistant ones, except for the most resistant strain, which accumulated an elevated amount of antibiotic. The FRAP/ciprofloxacin accumulation ratio of the antibiotic was lower in sensitive than in resistant strains. The most resistant strain exhibited the highest FRAP and presented a high catalase activity. There was oxidation of proteins in the presence of ciprofloxacin, with the carbonyl residues increasing in sensitive and resistant *S. aureus*. The degradation of carbonyls to AOPP in oxidized proteins was higher in the resistant than in sensitive strains. In conclusion, an increase in antioxidant capacity and a rapid oxidation of carbonyls to AOPP contributed to resistance to ciprofloxacin.

INTRODUCTION

The emergence of *Staphylococcus aureus* being resistant to antibiotics is a problem that affects different countries, with a notable increase in resistance to ciprofloxacin occurring during the last decade [1]. The single most common cause of resistance to fluoroquinolones is the appearance of mutations in the genes encoding the subunits of gyrase and topoisomerase IV [2]. However, other mechanisms involved in the acquisition of quinolone-resistance have also been described, such as the presence of efflux pumps [3].

The different aspects implicated in the effects of antibiotics on bacteria should be taken into consideration in order to understand the mechanism of action and resistance. For example, we have described that oxidative stress is associated with the actions of several antibiotics in bacteria, including ciprofloxacin [4–6]. In addition, it was previously shown that bacterial gyrase inhibitors such as synthetic quinolone antibiotics, promoted the formation of hydroxyl radical that contributed to cell death [7].

The vulnerability of the plasma protein to reactive oxygen species (ROS) and nitric oxide (NO) has been documented. Oxidative changes can lead to various functional consequences, such as inhibition of enzymatic activity, susceptibility to proteolysis and aggregation. The use of advanced oxidation protein products (AOPP) and carbonyls is important, as they provide markers of oxidative stress in order to estimate the degree of protein damage mediated by oxidants [8–10]. Related to this, it should be pointed out that the ability of bacteria to overcome oxidative stress depends on their antioxidant mechanisms, which may be able to reduce

the generation of ROS and ·NO to levels that are not harmful [11].

The purpose of this work was to investigate the oxidative stress generated by ciprofloxacin in the sensitive and resistant strains of *S. aureus*, the significance of the stimulus of ROS and NO in the oxidative damage of proteins, and the antioxidant capacity to defend against oxidative stress, with respect to the resistance to ciprofloxacin.

MATERIALS AND METHODS

Bacterial strains and susceptibility determination

Clinical strains were provided by Hospital Tránsito Cáceres de Allende (Buchardo 1250, Córdoba). *Staphylococcus aureus* (107, 8816, 98, 73, 22, 61, 13) were maintained by culture in trypticase soy broth (TSB) for 24 h at 37 °C. The minimum inhibitory concentration (MIC) was determined by using the standard tube dilution method. Strains coming from cultures of 24 h in Mueller-Hinton medium were diluted to10^6 CFU/mL, incubated for 10 min at 37 °C, and then ciprofloxacin was added at different concentrations (0.25–128 mg/L). Bacterial growth was observed at 24 h of incubation, following the indications of the Clinical and Laboratory Standards Institute [12]. The strains with MIC ≥4 mg/L were considered resistant to ciprofloxacin.

Reactive oxygen species (ROS) determination by CL

The bacterial density of *S. aureus* cultured in TSB for 24 h at 37 °C, was adjusted to $OD_{600}1$ in phosphate buffer (PBS) pH 7.8. Then, 0.1 mL of bacteria suspension was incubated with 0.1 ml of 75 mg/L lucigenin and 0.1 mL of PBS, followed by 0.1 mL of ciprofloxacin (0.45–1.8 mg/L), and the addition of 0.1 mL of dimethylsulphoxide. Controls were performed with bacteria in the absence of the antibiotic. The light emitted by ROS in a BioOrbit luminometer was expressed as relative light units (RLU) at different times in seconds, after subtraction of the background [13].

Nitric oxide

Bacterial suspension (0.25 mL) was added to 0.25 mL of 50 mM carbonate buffer pH 9 and 0.15 g of granulated cadmium for 30 min at room temperature. The reaction was stopped with 40 μL of 3.5 M NaOH. Samples were deproteinized with 0.4 mL 120 mM $ZnSO_4$ and centrifuged at 4472 ***g*** for 10 min. Then, 0.1 mL of the supernatant was mixed with 50 μL of 2% (w/v) sulfanilamide in 5% HCl and 50 μL of 0.1% (w/v) *N*-(1-naphthyl) ethylenediamine dihydrochloride. Spectrophotometry was performed at 540 nm. A standard curve was generated with sodium nitrite in concentrations from 0.1–1 mM [14].

Accumulation of ciprofloxacin

The strains (107, 8816, 98, 73, 22, 61, 13) grown on trypticase soy agar (TSA) were suspended in 50 mM PBS pH 7 (267 mg dry weight/mL). 0.1 mL of this suspension was diluted in 9.8 mL of PBS. Then, 0.1 mL of ciprofloxacin was added and incubated for 10 min at 37°C. The final concentration of the antibiotic in the sample was 10 mg/L. After 15 min of centrifugation at 10 000 × ***g***, the supernatant was discarded. The pellet was then washed and suspended in lysis buffer glycine–HCl (pH 3). Ciprofloxacin was determined by spectrofluorometry at 278 nm of excitation and 448.5 nm of emission [15].

Catalase determination

Bacterial suspension (0.1 mL) was added to the assay mixture of 2 mL of 0.2 M H_2O_2 and 2.5 mL of phosphate saline buffer for 10 min. One millilitre of the samples was taken periodically and mixed with 2 mL of the dichromate-acetic acid mixture, heated at 100 °C for 10 min and then maintained at 25 °C. The absorbance was determined at 570 nm. The quantity of H_2O_2 remaining was determined by means of a standard curve. Activity was expressed as units of CAT per mg of protein [16].

Ferrous reduction antioxidant potency

Staphylococcus aureus (0.080 mL) suspension was incubated with 0.125 mL of 3.1 mg/mL 2,4,6-tripyridyl-1,3,5-triazine (TPTZ) in 40 mM HCl, 0.125 mL of 5.4 mg/mL $FeCl_3{\cdot}6H_2O$ plus 1.25 mL of 300 mM acetate buffer (pH 3.6). Absorbances were read at 593 nm, and results were expressed as Fe^{+2} μmol/L × 10^{11} CFU/mL [17,18].

Carbonyl residues

Staphylococcus aureus suspensions were prepared from cultures of 18 h at 35 °C in triptein soya broth. Three millilitres of the sample was incubated with 0.5 mL of ciprofloxacin (at a sub-MIC concentration for each strain) or phosphate saline buffer (control) for 2–4 h. Then, 1 mL of the samples were treated with 1 mL of 0.1% 2,4-dinitrophenylhydrazine (2,4-DNPH) in 2 M HCl for 1 h. The proteins were precipitated in 5% trichloroacetic acid (TCA), centrifuged 20 min at 10 000 ***g***, and the supernatant discarded. Samples were

extracted three times with 1 mL ethanol-ethylacetate (1:1, v/v) to remove any remaining residual of DNPH. The precipitate was dissolved in 6 M guanidine hydrochloride solution in phosphate saline buffer pH 7.5 and incubated for 30 min at 37 °C. The insoluble debris was removed by centrifugation and the absorbance was measured at 364.5 nm. Results were expressed as mM of residues of carbonyl per mg protein and calculated using a molar extinction coefficient of 22/mol/cm for aliphatic hydrazones. All tests were performed in triplicate [19].

Determination of advanced oxidation protein products

Staphylococcus aureus suspensions were prepared from cultures of 18 h at 35 °C in triptein soya broth. 5 mL of the sample were incubated with 0.5 mL of ciprofloxacin (at a sub-MIC concentration for each strain) or phosphate saline buffer (control) for 2 and 4 h. Then, 1 mL of the samples or 1 mL of 50 μM chloramine T (standard) was treated with 50 μL of 1.16 M IK and 0.1 mL of acetic acid. The blank contained 1 mL of phosphate saline buffer, 50 μL of 1.16 M IK and 0.1 mL of acetic acid.

The absorbance of the reaction mixture was immediately read at 340 nm. The chloramine-T absorbance at 340 nm was linear within the range 0–100 mM. AOPP concentrations were expressed as micromoles per litre of chloramine-T equivalents [19].

Protein determination

The quantity of protein in bacterial suspensions was determined by the Folin-Ciocalteau assay [20].

RESULTS

The CL assays indicated a high degree of oxidative stress in the three sensitive strains in the presence of ciprofloxacin, with increases from 5- to 38-fold in the production of ROS with respect to strains without antibiotic, while the four resistant strains exhibited 0.7- to 2-fold increases in ROS. The response varied according to the concentration used. There was stimulation of ROS until the concentration of antibiotic produced excessive stress which decreased the production of ROS (*Figure 1*). Strain 98 suffered an important oxidative stress, because the maximum stimulus was obtained with 0.45 mg/L of antibiotic, while 107 and 8816 reached their maximum values (0.677 and 0.578 RLU, respectively) with 0.9 and 0.45 mg/L of ciprofloxacin. The rise in ROS was immediate (1 s) when the antibiotic was added, even at non-bactericidal concentrations.

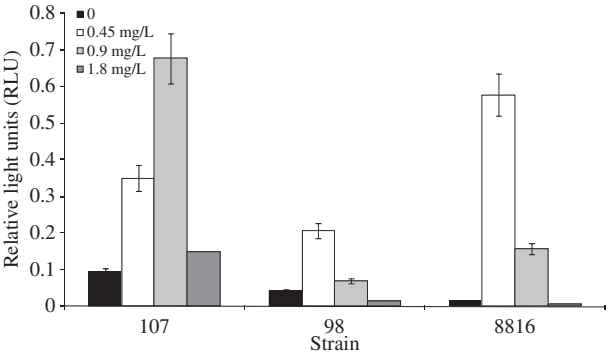

Figure 1 Increase of ROS production in *S. aureus* sensitive to ciprofloxacin. The strains were incubated at different concentrations of ciprofloxacin (0–1.8 mg/L).

The increase in ROS was lower in resistant strains (*Figure 2*) than in sensitive ones, without an obvious maximum value, even at the highest concentration of the assayed antibiotic (1.8 mg/L).

The determination of NO in bacteria showed that the mean ± SEM was higher in sensitive (4.9 ± 0.4 μM/mg protein) than in resistant strains (2.1 ± 0.3 μM/mg protein) ($P < 0.05$) (*Figure 3*).

Sensitive strains accumulated higher amounts of ciprofloxacin than resistant ones (*Figure 4*), but strain 73 presented a singular behaviour. Although this strain was the most resistant one, it accumulated a high amount of antibiotic as in the case of the most sensitive strains. The antioxidant capacity was investigated to try to explain the low stimulus of ROS and NO in spite of the high accumulation of ciprofloxacin in strain 73.

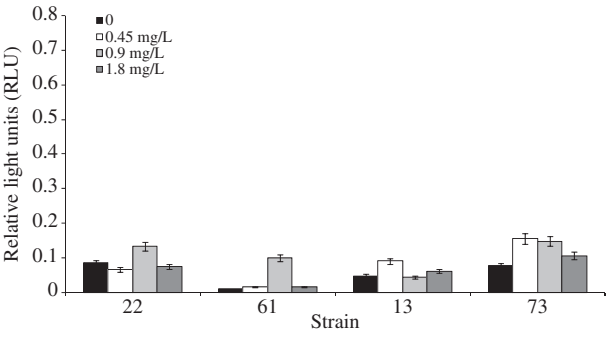

Figure 2 Reactive oxygen species (ROS) production in *S. aureus* resistant to ciprofloxacin. The strains were incubated at different concentrations of ciprofloxacin (0–1.8 mg/L).

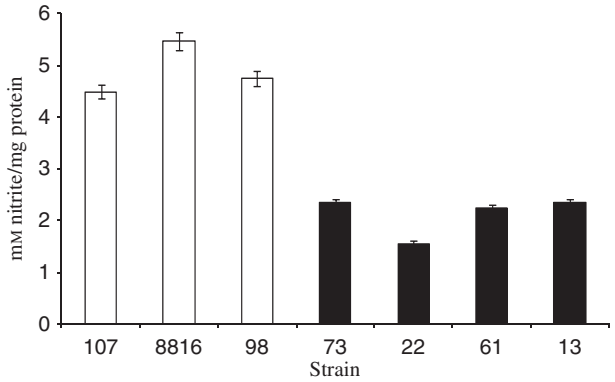

Figure 3 Nitric oxide level in sensitive (white bars) and resistant strains (black bars) in the presence of 0.45 mg/L of ciprofloxacin.

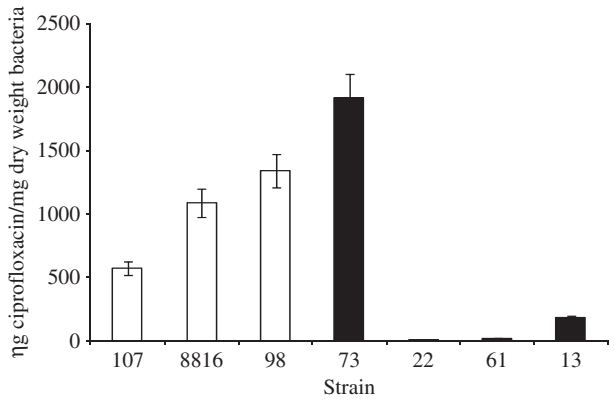

Figure 4 Accumulation of ciprofloxacin in sensitive (white bars) and resistant strains (black bars) of *S. aureus*.

The values of the antioxidant capacity of each strain are shown in *Table I*. It was found that the mean ferrous reduction antioxidant potency (FRAP) was 402 Fe^{+2} $\mu M \times 10^{11}$CFU/mL in sensitive strains and 1446 Fe^{+2} $\mu M \times 10^{11}$ CFU/mL in resistant bacteria, with strain 73 exhibiting the highest FRAP (3074 Fe^{+2} $\mu M \times 10^{11}$ CFU/mL). The FRAP/ciprofloxacin accumulation ratio was lower in sensitive (0.36 ± 0.1) than in resistant strains (52.3 ± 9.3), indicating that sensitive bacteria presented a poor defence against the rise in ROS generated by the accumulation of the antibiotic.

The CAT activity of sensitive strains (230 ± 1 U CAT/mg protein) was lower than that of the resistant ones (1071 ± 15 U CAT/mg protein), with strain 73 presenting one of the highest values of CAT activity (Table I).

Oxidation of proteins by ciprofloxacin was observed in all strains. Carbonyl residues increased to a mean value of 372 ± 5 mM/mg protein in sensitive *S. aureus*, within 2 h of incubation with ciprofloxacin (*Table II*), while the mean value of carbonyls for the resistant strains was lower (237 ± 5 mM/mg protein).

Table I Susceptibility to ciprofloxacin, antioxidant capacity, and catalase activity in sensitive and resistant strains of *S. aureus*.

	MIC mg/L	FRAP antioxidant capacity ± SEM (μM $Fe^{+2}/10^{11}$ CFU/mL)	CAT catalase ± SEM (U/mg protein)
Sensitive strains			
107	2	512 ± 128	124.62 ± 0.26
8816	2	175 ± 81	500.49 ± 0.26
98	1	30 ± 3	64.55 ± 0.65
Resistant strains			
73	128	3074 ± 1045	1733.24 ± 44.58
22	32	1459 ± 160	1940.11 ± 1.85
61	32	817 ± 91	468.05 ± 12.77
13	8	434 ± 35	144.48 ± 0.58

Advanced oxidation protein products (AOPP) also increased in the presence of the antibiotic, with the oxidation of carbonyl to AOPP being the most pronounced in resistant strains. The AOPP/carbonyl ratio was two- to sevenfold higher in resistant strains than in susceptible ones, when these were incubated with ciprofloxacin, with this ratio being time-dependent. The degradation of carbonyls was also higher in resistant strains than in sensitive ones.

DISCUSSION

The mechanisms involved in the resistance to ciprofloxacin can best be interpreted by considering the different aspects implicated in the antibacterial mechanism of action. In this way, we have demonstrated that ciprofloxacin, ceftazidime, piperacillin and chloramphenicol can generate an increase of superoxide anion in bacterial species with different degrees of defence against oxidative metabolism [5]. Also, a relationship was observed between resistance to oxidative stress and the concomitant resistance to antibiotics in biofilms of different bacteria species [21].

It was demonstrated previously, that oxidative stress was induced in bacteria exposed to rifampicin. This activated a global regulator, the sigma factor, which controls the general stress response and protects against multiple stress conditions [22]. Moreover, resistance to other antibiotics was found to be related to resistance to oxidative stress by up-regulation of σ^{β} [23]. Recently, it was postulated that antioxidants could contribute to the higher resistance to antifungals observed in *Candida* [24].

Table II Relationship between AOPP and carbonyl levels in sensitive and resistant strains of *S. aureus* at subMIC concentrations of CIP determined at different times.

	Carbonyl (mM/mg protein)		AOPP (mM chloramine T)		Ratio AOPP/Carbonyl		
	2 h	4 h	2 h	4 h	0 h	2 h	4 h
Sensitive strains							
107	359 ± 7	146 ± 3	43.2 ± 2.5	41.3 ± 2.2	0.03	0.12	0.28
8816	540 ± 5	291 ± 3	25.4 ± 1.3	74.6 ± 3.5	0.02	0.04	0.25
98	217 ± 3	187 ± 2	5.3 ± 0.8	1.1 ± 0.1	0.08	0.02	0.01
Resistant strains							
73	105 ± 2	63 ± 1	207 ± 2	196 ± 4	0.08	1.97	3.11
22	186 ± 3	141 ± 3	139 ± 3	129 ± 2	0.01	0.75	0.91
61	364 ± 5	258 ± 3	98 ± 1	101 ± 2	0.03	0.27	0.40
13	292 ± 5	285 ± 4	312 ± 4	272 ± 3	0.10	1.06	0.95

On the basis of all these previous results, oxidative stress and accumulation were investigated in the present work by considering that micro-organisms develop defences in response to oxidative injury. The results obtained indicated that oxidative stress was associated with the effect of ciprofloxacin, since the analysis of ROS and NO demonstrated that resistant strains presented a low stimulus of these two promoters of oxidative damage. Although, resistant strains had a low accumulation of ciprofloxacin; strain 73, the resistant strain with highest MIC, accumulated antibiotic but did not increase either ROS or NO as consequence of its elevated CAT and FRAP. Therefore, as a simple analysis of accumulation of ciprofloxacin could not explain the behaviour of all strains, it was necessary to consider the antioxidant defences in order to understand the resistance to ciprofloxacin. Related to this, another investigation reported a protective effect of antioxidants against the antibacterial activity of fluoroquinolones and aminoglycosides in *Escherichia coli* [25].

In addition to the high antioxidant properties of resistant strains, must be point out the fast degradation of carbonyl to AOPP in these strains should be taken into account. It is important to note that carbonyls are intermediate products in the oxidation of proteins, whereas AOPP are the products of the advanced oxidation protein, which leads to renovation of the proteins.

The oxidative stress generated by ciprofloxacin provoked an irreversible degradation of the vital macromolecules, such as the bacterial proteins. The formation of carbonyl groups was higher in sensitive strains than in resistant ones, because these after ones had a fast turnover of proteins as a natural defensive mechanism against oxidative damage. Related to this, there was a correlation between the reduction of carbonyl groups and an increase in AOPP.

In conclusion, the mechanisms of resistance to ciprofloxacin implicate various factors. The concept of high efflux as a cause of resistance to this fluoroquinolone should be investigated, since resistant bacteria such as strain 73 can counteract their low efflux of antibiotic by means of high FRAP and CAT, which together protect against oxidation by CIP and cause a fast and protective conversion of carbonyl to AOPP. Finally, a better understanding of the oxidative stress response of microbial pathogens against oxidative injury caused by antibiotics may aid the future development of antimicrobial treatment and pharmacological strategies.

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REFERENCES

1 Bearden D.T., Danziger L.H. Mechanisms of action and resistance to quinolonas. Pharmacotherapy (2001) **21** 224–232.
2 Black M.T., Stachyra T., Platel D. Mechanism of action of the antibiotic NXL101, a novel nonfluoroquinolone inhibitor of bacterial type II topoisomerases. Antimicrob. Agents. Chemother. (2008) **52** 3339–3349.
3 Piddock L.J., Jin Y.F., Webber M.A. Novel ciprofloxacin-resistant, nalidixic acid-susceptible mutant of *Staphylococcus aureus*. Antimicrob. Agents. Chemother. (2002) **46** 2276–2278.
4 Becerra M.C., Albesa I. Oxidative stress induced by ciprofloxacin in *Staphylococcus aureus*. Biochem. Biophys. Res. Commun. (2002) **297** 1003–1007.

5 Albesa I., Becerra M.C., Battan P.C., Páez P.L. Oxidative stress involved in the antibacterial action of different antibiotics. Biochem. Biophys. Res. Commun. (2004) **317** 605–609.
6 Becerra M.C., Páez P.L., Laróvere L.E., Albesa I. Lipids and DNA oxidation in *Staphylococcus aureus* as a consequence of oxidative stress generated by ciprofloxacin. Mol. Cell. Biochem. (2006) **285** 29–34.
7 Kohanski M.A., Dwyer D.J., Hayete B. A common mechanism of cellular death induced by bactericidal antibiotics. Cell (2007) **130** 797–810.
8 Capeillere-Blandin C., Gausson V., Descamps-Latscha B., Witko-Sarsat V. Biochemical and spectrophotometric significance of advanced oxidized protein products. Biochim. Biophys. Acta (2004) **1689** 91–102.
9 Skvarilova M., Bulava A., Stejskal D., Adamovska S., Bartek J. Increased level of advanced oxidation products (AOPP) as a marker of oxidative stress in patients with acute coronary syndrome. Biomed. Papers (2005) **149** 83–87.
10 Gangemi S., Saija A., Tomaino A., et al. Protein carbonyl group content in patients affected by familiar chronic nail candidiasis. Mediators. Inflamm. (2003) **12** 247–249.
11 Bandow J.E., Brotz H., Hecker M. *Bacillus subtilis* tolerance of moderate concentrations of rifampin involves the sigma(B)-dependent general and multiple stress response. J. Bacteriol. (2002) **184** 459–467.
12 Clinical and Laboratory Standards Institute (CLSI) (formerly NCCLS): Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically; Approved Standard - Seventh Edition. CSLI document M7-A7. Clinical and Laboratory Standards Institute, Wayne, PA, USA, 2006.
13 Bromme H.J., Ebelt H., Peschke D., Peschke E. Alloxan acts as a prooxidant only under reducing conditions: influence of melatonin. Cell. Mol. Life Sci. (1999) **55** 487–493.
14 Aggarwal B.B., Mehta K. Determination and regulation of nitric oxide production from macrophages by lipopolysaccharides, cytokines, and retinoids. Meth. Enzymol. (1996) **269** 166–171.
15 McCaffrey C., Bertasso A., Pace J., Georgopapadakou N.H. Quinolone accumulation in *Escherichia coli, Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Antimicrob. Agents Chemother. (1992) **36** 1601–1605.
16 Andrews G.P., Martin S.E. Catalase activity during the recovery of heat-stressed *Staphylococcus aureus* MF-31. Appl. Environ. Microbiol. (1979) **38** 390–394.
17 Benzie I.F., Strain J.J. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Meth. Enzymol. (1999) **299** 15–27.
18 Aiassa V., Barnes A.I., Albesa I. Action of ciprofloxacin on planktonic bacteria and biofilm of *Proteus mirabilis*. Biofilms (2006) **3** 11–17.
19 Witko-Sarsat V., Friedlander M., Nguyen Khoa T., et al. Advanced oxidation protein products as novel mediators of inflammation and monocyte activation in chronic renal failure. J. Immunol. (1998) **161** 2524–2532.
20 Stauffer C. E. A linear standard curve for the Folin Lowry determination of protein. Anal. Biochem. (1975) **69** 646–648.
21 Battan P.C., Barnes A.I., Albesa I. Resistance to oxidative stress caused by ceftazidime and piperacillin in a biofilm of *Pseudomonas*. Luminescence (2004) **19** 265–270.
22 Bandow J.E., Brotz H., Hecker M. *Bacillus subtilis* tolerance of moderate concentrations of rifampin involves the sigma(B)-dependent general and multiple stress response. J. Bacteriol. (2002) **184** 459–467.
23 Bischoff M., Berger-Bächi B. Teicoplanin stress-selected mutations increasing sigma(B) activity in *Staphylococcus aureus*. Antimicrob Agents Chemother. (2001) **45** 1714–1720.
24 Seneviratne J., Wang Y., Jin L. *Candida albicans* biofilm formation is associated with increased anti-oxidative capacities. Proteom (2008) **8** 936–947.
25 Goswami M., Jawali N. Glutathione-mediated augmentation of ß-lactam antibacterial activity against *Escherichia coli*. J. Antimicrob. Chemother. (2007) **60** 184–185.