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Effects of Some Antibiotics on Human Erythrocyte 6-Phosphogluconate Dehydrogenase: An *in vitro* and *in vivo* Study

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The *in vitro* and *in vivo* effects of some antibiotics on human erythrocyte 6-phosphogluconate dehydrogenase were investigated. Human erythrocyte 6-phosphogluconate dehydrogenase was purified with ammonium sulphate precipitation, 2′,5′ ADP-Sepharose 4B affinity and gel filtration chromatography. Some antibiotics (netilmicin sulphate, cefepime, amikacin, ısepamycin, chloramphenicol, ceftazidim, teicoplanin, ampicillin, ofloxacin, levofloxacin, cefotaxime, penicillin G, gentamicin sulphate, ciprofloxacin) inhibited enzyme activity *in vitro* but others (cefozin, decefin, streptomycin, combisid, and meronem) were devoid of inhibitory effects. For the drugs having low IC_{50} values (netilmicin sulphate and cefepime), *in vivo* studies were performed in rats. Netilmicin sulphate at 15-mg/kg inhibited enzyme activity significantly ($p < 0.001$) 1 h, 2 h, and 3 h after dosing and cefepime at 200-mg/kg very significantly ($p < 0.001$) inhibited the enzyme 1 h and 2 h after dosing. Netilmicin sulphate and cefepime inhibited rat erythrocyte 6-phosphogluconate dehydrogenase both *in vivo* and *in-vitro*.

Keywords: 6-Phospogluconate dehydrogenase; Erythrocytes; Human; Antibiotics; Inhibitors

INTRODUCTION

6-Phosphogluconate dehydrogenase (E.C.1.1.1.44; 6PGD), the third enzyme in the pentose phosphate metabolic pathway, catalyzes the conversion of 6-PGA and $NADP^+$, to D-ribulose-5-phosphate and NADPH which protects the cell against oxidizing agents by producing reduced glutathione (GSH).[1,2] NADPH is also a coenzyme participating in the synthesis of a number of biomolecules such as fatty acids, steroids, and some amino acids.[3,4] In the case of lack of NADPH, the concentration of GSH in living cells declines, resulting in cell death. GSH is indirectly produced by 6PGD, therefore, 6PGD can be defined as an indirect antioxidant enzyme.[4,5] Many agents are known to activate or inhibit enzymes *in vitro* and *in vivo*,[6–9] so affecting metabolic pathways. Inhibition of 6PGD leads to decreased NADPH and GSH which will cause cell damage especially in older erythrocytes, resulting in some problems in living cells.[6–8] No reports could be found in the literature on the *in vitro* and *in vivo* effects of some drugs on human erythrocyte 6PGD although vitamin C has been reported to stimulate 6PGD.[10]

This study was aimed at purifying human erythrocyte 6PGD, and to determination of effects of some commonly used antibiotics on human red blood cell 6PGD activity.

MATERIALS AND METHODS

Materials

6-PGA, $NADP^+$, Tris, and the other chemicals were from Sigma Chem. Com. and the drugs were purchased from Hoechst Marian Roussel (Turkey).

Activity Determination

Enzymatic activity was measured by Beutler's method.[11] One unit of enzyme (EU) activity was

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defined as the amount of enzyme reducing 1 μmol $NADP^+$ per 1 min at 25°C, pH 8.0.

Preparation of the Hemolysate and Hemoglobin Estimation

Fresh human blood samples were collected in tubes containing EDTA, then centrifuged (15 min, 2,500 × g) and plasma and buffy coat (leucocytes) were removed. The packed red cells were washed three times with KCl (0.16 M) and hemolyzed with 5 volume of ice-cold water and then centrifuged (4°C, 10,000 × g, for 30 min) to remove the ghosts and intact cells. Hemoglobin (Hb) concentration in the hemolysate was determined by the cyan-methaemoglobin method.[11–13]

Ammonium Sulphate Precipitation

The hemolyzate was subjected to precipitation with ammonium sulphate (between 35% and 65%). Enzyme activity was determined both in the supernatant and in the precipitate for each respective precipitation. The precipitated enzyme was dissolved in phosphate buffer (50 mM; pH = 7.0). The resultant solution was clear, and contained partially purified enzyme.

Purification of the 6-PGD

For purification of 6-PGD, the ammonium sulphate fraction (35–65%) of the homogenate obtained previously was loaded onto a 2′, 5′-ADP Sepharose 4B affinity column and the flow rate was adjusted to 20 ml/h. The column was then sequentially washed with a 25 ml buffer of 0.1 M K-acetate and 0.1 M K-phosphate, (pH 6.0) and 25 ml of a buffer of 0.1 M K-acetate and 0.1 M K-phosphate (pH 7.85). The wash with the second buffer was continued until the final absorbance difference became 0.05. Elution of the enzyme was carried out with a mixture containing 80 mM K-phosphate, 80 mM KCl, 5 mM $NADP^+$ and 10 mM EDTA (pH 7.85). Enzyme activity was measured in all fractions, and the activity-containing fractions were pooled, then dialyzed in 50 mM K-acetate + 50 mM K-phosphate buffer (500 ml, pH 7.0) for 2 h with two changes of buffer. All procedures were performed at 4°C.[12,14]

Sephadex G-200 Gel Filtration Chromatography

5 g of dry Sephadex G-200 was incubated in the distilled water at 90 °C for 5 hours. After removal of the air in the gel, it was loaded onto a column (2 × 50 cm). Flow rate was adjusted to 15 mL/h by means of peristaltic pump. The column was equilibrated with 50 mM Tris-HCl, 50 mM KCl buffer, pH: 7.3 until the final absorbance difference became zero at 280 nm and pH value became same with that of equilibration buffer. The dialysed from affinity chromatography column was mixed with glycerol at the ratio of 5%. The final sample was loaded onto the column and each elution was collected in Eppendorf tubes as 2 mL. The absorbance values were determined at 280 nm and at 340 nm in each fractions. Active fractions were lyophilized and stored at − 85 °C for the use in *in vitro* studies.

Protein Determination

The protein content in all samples was quantified spectrophotometrically at 595 nm according to Bradford's method,[15] using bovine serum albumin as standard.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The control of enzyme purity was carried out using Laemmli's procedure[16] with 3% and 8% acrylamide concentrations for running and stacking gel, respectively. *E. coli* β-galactosidase (116,000), rabbit phosphorylase B (97,400), bovine albumin (66,000), chicken ovalbumin (45,000), and bovine carbonic anhydrase (29,000) were used as standards (Sigma: MW-SDS-200).

In vitro Drug Effect

In order to determine the effects of some antibiotics on human 6PGD, concentrations of gentamicin sulphate (4.15–33.20 mM), amikacin sulphate (4.26–14.20 mM), netilmicin sulphate (3.47–20.82 mM), ısepamycin (21.90–175.20 mM), chloramphenicol (10.30–77.25 mM), ceftazidim (1.55–7.75 mM), teicoplanin (7.2–96 mM), ampicillin (31.80–150 mM), ofloxacin (0.11–0.412 mM), cefotaxime (1.50–15 mM), levofloxacin (0.70–4.20 mM), cefepime (1.04–10.39 mM), penicillin G (41.94–419.45 mM), and ciproflaxacin (0.12–1.20 mM), were added to the purified enzyme. The enzyme activity was measured and an experiment in the absence of drug was used as control (100% activity). The IC_{50} values were obtained from activity (%) *vs.* drug concentration plots.

In vivo Drug Effect

In vivo studies were conducted only for those compounds having low IC_{50} values. i.e. netilmicin sulphate and cefepime. Each drug-treated group comprised six rats (180 ± 20 g) which were kept under special conditions (in a windowless room, at of 22°C, with light on for 14 h) for 2 months. Before intramuscular drug injection, control blood samples (0.5 ml of whole blood with EDTA) were obtained from the animals. Then 15 mg/kg of netilmicin sulphate was injected into one group and 200 mg/kg of cefepime into the other. Blood samples were

taken from each group 1, 2, and 3 h after injection. Hemolysates were prepared from all blood samples as mentioned above. Hemoglobin levels and 6-PGD activity were determined in these hemolysates.

Statistical Analysis

The mean values and standard deviations of the results were determined using student *t*-test for statistical evaluation of the difference, with $p < 0.001$ considered very significant.

RESULTS

The purification of the enzyme led to a specific activity of 1.886 EU/mg proteins, a yield of 49.57% and a purification coefficient of 725 (Table I). Figure 1 shows the SDS-PAGE for the purity of the enzyme.

Figure 2 shows the *in vitro* inhibitory effects of four typical antibiotics (netilmicin sulphate, cefepime, amikacin, and isepamycin) on enzyme activity. The IC_{50} values were: netilmicin sulphate (3.372 mM), cefepime (4.213), amikacin (3.630), isepamycin (30.528), chloramphenicol (36.283), ceftazidim (2.110), teicoplanin (21.521), ampicillin (36.282), ofloxacin (0.118), levofloxacin (2.142), cefotaxime (3.559), penicillin G (161.162), gentamicin sulphate (4.932), ciprofloxacine (0.271).

The results of the *in vivo* effects of netilmicin sulphate and cefepime are presented in Table II. In the netilmicin sulphate-treated group of animals, the control enzyme activity was 3.627 ± 0.419 EU/gHb, while the respective values determined 1, 2 and 3 h after drug administration were 1.195 ± 0.115 EU/gHb, 0.354 ± 0.056 EU/gHb and of 1.821 ± 0.123 EU/gHb. In the cefepime-treated group of animals, the control enzyme activity was 3.893 ± 0.260 EU/gHb, and 1, 2, and 3 h after drug treatment it was 1.197 ± 0.365 EU/gHb, 0.362 ± 0.077 EU/gHb and 3.628 ± 0.350 EU/gHb respectively.

DISCUSSION

Human 6PGD from erythrocytes was purified in this study by hemolysate preparation, ammonium sulphate precipitation, 2′,5′-ADP Sepharose 4B affinity chromatography and gel filtration chromatography. The purified preparation was characterized with a specific activity of 1.886 EU/mg protein, a yield of 49.57% and a purification coefficient of 725. These figures tend to validate the procedure used in the study. The SDS-PAGE shows the high purity of the enzyme.

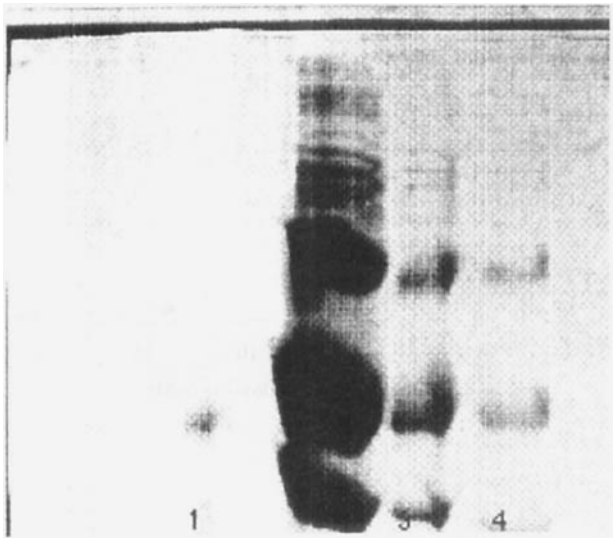

FIGURE 1 SDS-PAGE bands of 6PGD (Lane 1: 6PGD enzyme, Lane 2, 3, 4: standard proteins; *E.Coli* β-galactosidase (116,000), rabbit phosphorylase B (97,400), bovine albumin (66,000), chicken ovalbumin (45,000), and bovine carbonic anhydrase (29,000) were used as standards (Sigma: MW-SDS-200).

The antibiotics which were used this study are commonly used clinically throught the world. However, some are associated with side effects such as nephrotoxicity, ototoxicity, neurotoxicity, fever, bone marrow depression, fever, allergy, epidermal eruption and hemolytic anemia.[17] Ampicillin inhibits human red cells G6PD,[18] sheep liver G6PD,[19] rat 6PGD,[9] and human CA,[20] netilmicin sulphate inhibits human red cells G6PD,[18] rat 6PGD[9] and amikacin inhibits sheep liver G6PD,[21] sheep red blood cells G6PD,[18] and rat 6PGD.[9] In addition, it has been reported that ampicillin, netilmicin and amikacin inhibit the rat 6PGA and G6PD *in vivo*.[9,18]. We could not find any

TABLE I Purification scheme for 6-phosphogluconate dehydrogenase from human erythrocytes

Purification step	Activity (EU/ml)	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Total activity (EU)	Specific activity (EU/mg)	Yield (%)	Purification factor
Haemolysate	0.1028	50	39.51	1,975.5	5.14	0.0026	100	1
Ammonium sulphate precipitation (35–65)%	0.228	20	46.89	937.8	4.56	0.0048	88.71	1.846
2′,5′- ADP sepharose 4B affinity chromatography	0.2685	15	0.224	3.36	4.0275	1.198	78.35	460.76
Sephadex G 200 Gel Filtration	0.166	14	0.088	2.38	2.548	1.886	49.57	725.38

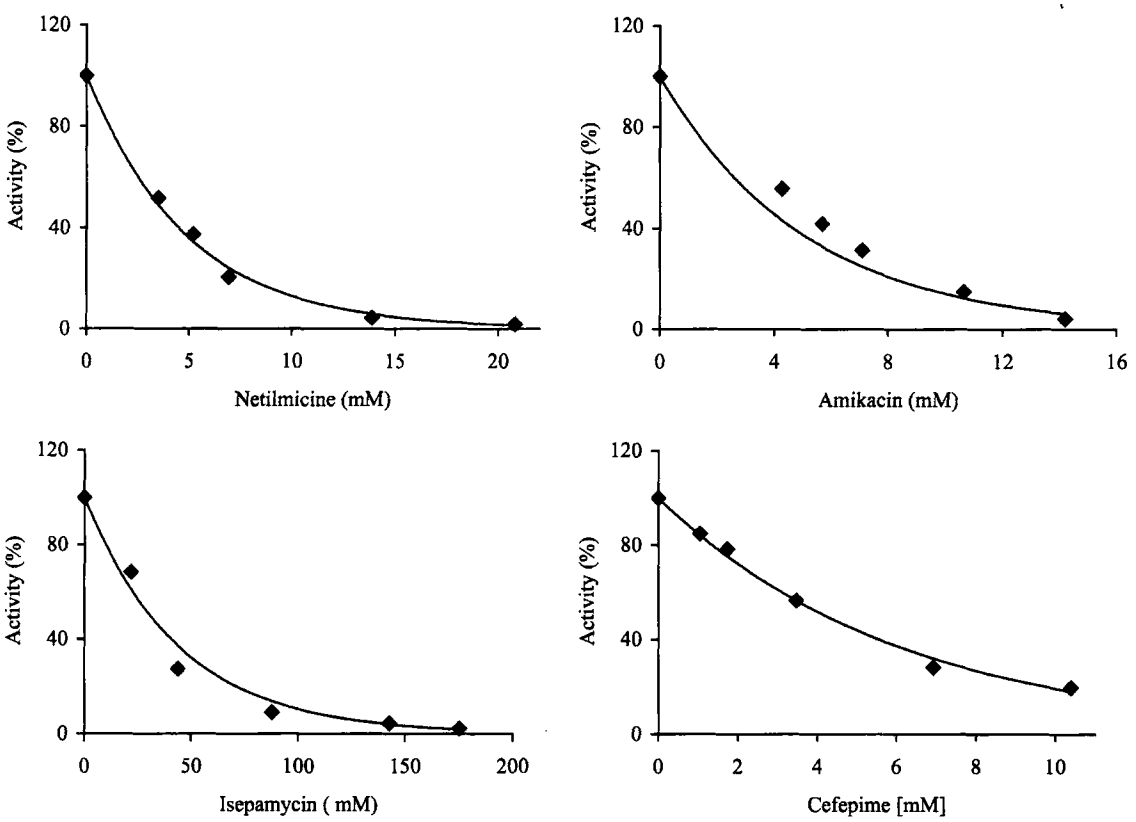

FIGURE 2 Activity (%) *vs.* antibotics concentrations regression analysis graphs for 6PGD in the presence of different antibotic concentrations.

TABLE II K_i values obtained from Lineweaver-Burk graphs for 6PGD inhibitors

Inhibitor	[I] (mM)	K_i (mM)	K_i (Mean value)	Inhibition type
Netilmicin	0.694	0.775	0.939 ± 0.152	Competitive
	2.082	1.075		
	5.205	0.968		
Cefepime	1.04	3.104	2.579 ± 0.810	Noncompetitive
	2.08	2.988		
	3.12	1.646		

previous reports related to the effects of other drugs on 6PGD activity. In the present study, the effect of some commonly used drugs on 6PGD has been investigated *in vitro* and in an animal model *in-vitro*.

Amikacin, ısepamycin, chloramphenicol, ceftazidim, teicoplanin, ampicillin, ofloxacin, levofloxacin, cefotaxime, penicillin G, gentamicin sulphate, and ciprofloxacin inhibit 6PGD *in vitro* (Figure 2) and netilmicin sulphate and cefepime are inhibitory both *in vitro* and *in vivo* (Table III).

From the *in vitro* studies, it was clear that 6PGD was more strongly inhibited by netilmicin sulphate, cefepime, amikacin, ceftazidim, ofloxacin, levofloxacin, cefotaxime, gentamicin sulphate and ciprofloxacin of the drugs tested. Of these drugs, netilmicin sulphate ($K_i = 0.939$ mM) and cefepime (2.58) which are commonly used in clinical medicine were also studied *in vivo* (Figure 3, Table II and III).

The *in vivo* studies showed that netilmicin sulphate inhibited the enzyme by ~91% after 2 hours ($p < 0.0001$) and ~50% after 3 hours ($p < 0.0001$) and cefepime inhibited the enzyme by ~91% after 2 hours ($p < 0.0001$) while the effects observed after 3 hours were not statistically significant (Table III). The *in vitro* and *in vivo* results support each other. The half-lives of netilmicin sulphate and cefepime are 2–3 h,[17] which accounts for the fact that enzyme activity has begun to rise after the third hour due to elimination of the drug.

TABLE III *In vivo* effects of netilmicin sulphate and cefepime on rat red blood cell 6-phosphogluconate dehydrogenase activity ($n = 6$)

Drug	Time (h)	$\bar{X}$ ± SD (EU/gHb)	P
Netilmicin sulphate	Control	3.627 ± 0.419	–
	1	1.195 ± 0.115	<0.0001
	2	0.354 ± 0.056	<0.0001
	3	1.821 ± 0.123	<0.0001
Cefepime	Control	3.893 ± 0.260	–
	1	1.197 ± 0.365	<0.0001
	2	0.362 ± 0.077	<0.0001
	3	3.628 ± 0.350	>0.05

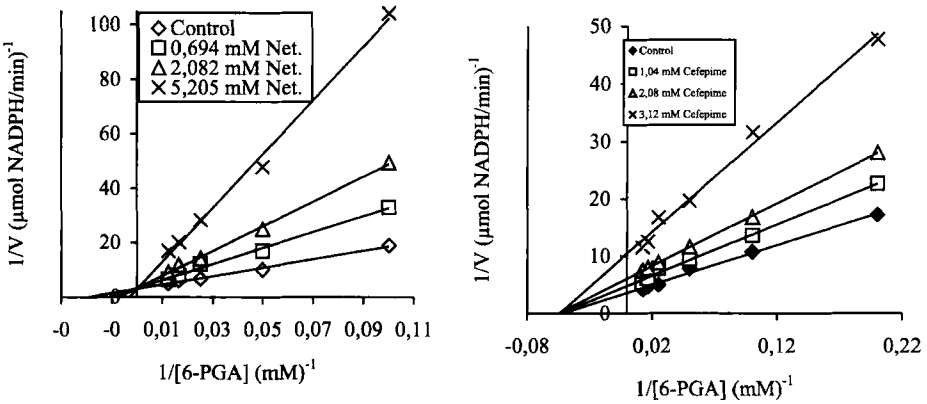

FIGURE 3 Lineweaver-Burk graphs using 3 different netilmicin and cefepime concentrations for determination of K_i.

The doses of netilmicin sulphate (15 mg/kg) and cefepime (200 mg/kg) used here are consistent with previous studies, since the clinical doses of 37.5–200 mg/kg and 20–2000 mg/kg have been reported for netilmicin and cefepime respectively.[22–28]

The use of these antibiotics can cause serious adverse effects such as hemolytic effects, which may move fatal. If it is required to give netilmicin sulphate and cefepime to patients, their dosage should be very well controlled to decrease hemolytic and other side effects.

References

[1] Bianchi, D., Bertrant, O., Haupt, K. and Coello, N. (2001) *Enz. Microb. Technol.* **28**, 754–759.
[2] Lehninger, A.L., Nelson, D.L. and Cox, M.M. (2000) *Principles of Biochemistry*, 2nd edn. (Worth Publishers Inc., New York), pp 558–560.
[3] Kahler, S.G. and Kirkman, H.N. (1983) *J. Biol. Chem.* **258**, 717–718.
[4] Srivastava, S.K. and Beutler, E. (1989) *Biochem. J.* **119**, 353.
[5] Kozar, R.A., Weibel, C.J., Cipolla, J., Klein, A.J., Haber, M.M., Abedin, M.Z. and Trooskin, S.Z. (2000) *Crit. Care Med.* **28**, 2486–2491.
[6] Beutler, E. (1994) *Blood* **84**, 3613–3636.
[7] Edward, E. and Morse, M.D. (1988) *Ann. Clin. Lab. Sci.* **18**, 13–18.
[8] Jacobasch, G. and Rapoport, S.M. (1996) *Mol. Asp. Med.* **17**, 143–170.
[9] Çiftçi, M., Beydemir, S., Yılmaz, H. and Bakan, E. (2002) *J. Pharmacol.* **54**, 275–280.
[10] Puskas, F., Gergely, P.J., Banki, K. and Perl, A. (2000) *FASEB J.* **14**, 1352–1361.
[11] Beutler, E. (1971) *Red Cell Metabolism Manual of Biochemical Methods* (Academic Press, London), p 12.
[12] Ninfali, P., Orsenigo, T., Barociani, L. and Rapa, S. (1990) *Biochem. Biotech.* **20**, 297–309.
[13] Shreve, D.S. and Levy, H.R. (1977) *Biochem. Biophys. Res. Comm.* **78**, 1369–1375.
[14] Morelli, A., Benatti, U., Gaetani, G.F. and De Flora, A. (1978) *Proc. Natl Acad. Sci. USA* **75**, 1979–1983.
[15] Bradford, M.M. (1976) *Anal. Biochem.* **72**, 248–251.
[16] Laemmli, D.K. (1970) *Nature* **227**, 680–683.
[17] Kayaalp, S.O. (1998) *Rasyonal tedavi yönünden tıbbi farmakoloji (Turkish)* (Hacettepe-Tas Yayıncılık, Ankara).
[18] Çiftçi, M., Küfrevioğlu, Ö.İ., Gündoğdu, M. and Özmen, İ. (2000) *Pharmacol. Res.* **41**(1), 109–113.
[19] Beydemir, S., Çiftçi, M. and Küfrevioğlu, Ö.İ. (2002) *J. Enzy. Inhib. Med. Chem.* **17**(4), 271–277.
[20] Beydemir, S., Çiftçi, M. and Küfrevioğlu, Ö.İ. (2002) *Biol. and Pharm. Bull.* **25**(8), 966–969.
[21] Çiftçi, M., Türkoğlu, V. and Aldemir, S. (2002) *Vet. Med. Czech.* **47**(10–11), 283–288.
[22] Carlier, E. and Pujol, R. (1980) *Arch. Otorh.* **3**, 129–133.
[23] Josepovitz, C., Levine, R., Farruggella, T. and Kaloyanides, G.J. (1986) *Proc. Soc. Biol. Med.* **182**, 1–5.
[24] Mouedden, E.I., Laurent, G., Mingeot-Leclercq, M.P., Taper, H.S., Cumps, J. and Tulkens, P.M. (2000) *Antimicrob. Agents Chemother.* **44**, 665–675.
[25] Slighter, R.G., Montenaro, M.J., Fabian, R.J., Bhandari, J.C., Donikian, M.R. and Drobeck, H.P. (1984) *Fundam. Appl. Toxicol.* **4**, 558–567.
[26] Toubeau, G., Laurent, G., Carlier, M.B., Abid, S., Maldague, P., Heuson-Stiennon, J.A. and Tulkens, P.M. (1986) *Lab. Investig.* **54**, 385–393.
[27] Kai, S., Kohmura, H., Ishikawa, K., Kawano, S., Sakai, A., Kuroyanagi, K., Kadota, T. and Takahashi, N. (1992) *Jpn. J. Antibiot.* **45**(6), 642–660.
[28] Forgue, S.T., Shyu, W.C., Gleason, C.R., Pittman, K.A. and Barbhaiya, R.H. (1987) *Antimicrob. Agents Chemother.* **31**(5), 799–804.